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Fungal Contribution to Carbon and Nutrient Cycling in a Subtropical Freshwater Marsh

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The University of Southern Mississippi

FUNGAL CONTRIBUTION TO CARBON AND NUTRIENT CYCLING IN A
SUBTROPICAL FRESHWATER MARSH

by

Rong Su

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

December 2014

ABSTRACT

FUNGAL CONTRIBUTION TO CARBON AND NUTRIENT CYCLING IN A SUBTROPICAL FRESHWATER MARSH

by Rong Su

December 2014

Despite the well-known occurrence of emergent standing litter in freshwater marshes, very little quantitative data is available concerning the functional role and contribution of fungal decomposers to litter decomposition or their impact on ecosystem scale carbon and nutrient cycling. In the present study, microbial respiration and seasonal biomass and production rates of fungal decomposers associated with standing plant litter were examined to assess the quantitative contribution of fungi to both fine scale litter decay processes and large-scale ecosystem carbon flow pathways in a subtropical *Typha domingensis* and mixed *Cladium jamaicense*/*Juncus roemarianus* freshwater marsh, respectively. In addition, the growth and biomass dynamics of fungal decomposers in standing litter were also examined in relation to changes in detrital nitrogen and phosphorus concentrations in order to assess the importance of fungi to nutrient cycling. When integrated over the annual study period, estimated cumulative fungal production in *T. domingensis* standing litter totaled 39 mgC/g initial detrital C, indicating that 10.5% of the observed *Typha* leaf carbon lost was assimilated into fungal biomass. Corresponding estimates of microbial respiration (CO₂ flux) totaled 133 mgC/g initial detrital C, providing evidence that a considerable amount of *Typha* leaf carbon is also mineralized by microbial communities, most likely fungi. When examined at the ecosystem scale, estimated annual fungal production within the mixed *C. jamaicense*/*J. roemarianus*

marsh totaled $90 \pm 4 \text{ gC/m}^2$, which was equivalent to $\sim 14\%$ of mean annual standing-dead litter mass ($643 \pm 104 \text{ gC/m}^2$). Estimated annual carbon flux from microbial respiration totaled $124 \pm 5 \text{ gC/m}^2$, representing $\sim 19\%$ of mean annual standing-dead litter mass. In addition to assimilating carbon, fungi also were a significant contributor to the transformation and assimilation of detrital nutrients. Using a fungal stoichiometric ratio of $\text{C}_{106}\text{N}_{16}\text{P}_1$, fungi inhabiting standing litter immobilized $>35\%$ the total detrital N and P during decomposition. Results obtained in this study provide compelling evidence that fungal decomposers can play a key role in the cycling of carbon and nutrients in freshwater marshes. Hopefully, these findings will extend our current conceptual understanding of biogeochemical pathways in freshwater marshes.

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A Dissertation
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CHAPTER I

INTRODUCTORY BACKGROUND AND RESEARCH NEED

Emergent freshwater marshes. Freshwater marshes are one of the most biologically productive ecosystems on earth, and are known for their high biodiversity and extensive food webs (Batzler and Sharitz 2006; Mitsch and Gosselink 2007; Keddy 2010). The growth of emergent vascular plants, such as *Typha*, *Juncus*, and *Cladium*, is common within freshwater marshes, where they often form dense monotypic stands that can effectively out-compete other plant species. These plants exhibit high rates of primary production (e.g., aboveground production typically $> 1 \text{ kgC/m}^2/\text{y}$) and frequently form most of the plant biomass produced on an annual basis (Wetzel and Howe 1999; Wetzel 2006; Mitsch and Gosselink 2007). Furthermore, because of their prolific growth and concomitant nutrient demands, these emergent plants also absorb and sequester large amounts of nutrients, such as nitrogen and phosphorus, and thus represent an important reservoir of nutrients (Mitsch and Gosselink 2007).

In freshwater marshes, most of the living plant biomass is not consumed via herbivory (e.g., Dvůrák and Imhof 1998; Wetzel and Howe 1999) but eventually enters the detrital pool following plant senescence and death, where heterotrophic microbial decomposers (bacteria and fungi) colonize, enzymatically degrade, and assimilate plant litter carbon and nutrients in amounts sufficient to bring about the breakdown and mineralization of plant matter. The growth and biomass production of microbial communities on decaying plant litter in turn serves as an important food resource for detritus feeding consumers (Cebrian 1999; Moore et al. 2004; Kulesza and Holomuski 2006; Hagen et al. 2012), where they facilitate the litter decomposition process through

their feeding activities. As a consequence, many characteristics related to carbon and nutrient cycling in freshwater marsh ecosystems are intimately coupled with, and influenced by, the decay activities of litter-associated microorganisms.

The high primary productivity of emergent marsh plants and their subsequent decomposition result in freshwater marshes functioning as: (1) major sources of organic (detrital) carbon and (2) major sites where nutrients, such as nitrogen and phosphorus, are stored and processed. Each of these roles are associated with the chemical composition and biomass production of the emergent vegetation, which in turn influences the composition, biomass, and activity of the inhabitant microbial communities and the decomposition rate of plant litter. Given the recognized importance of detritus in virtually every ecosystem (Moore et al. 2004; Hagen et al. 2012), I argue that an understanding of wetland ecological processes should include a quantitative examination of the conversion and flow of carbon and nutrients from plant detritus to and through litter-associated microbial decomposers.

Emergent plant decomposition. Despite the well-known occurrence and ecological importance of emergent plant litter in freshwater marshes, the natural phenology of its decomposition has rarely been examined (Newell 1993; Bärlocher 1997; Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008). In addition, the decomposition of emergent plant litter in freshwater marshes has rarely been examined in relation to the composition and dynamics (i.e., rates of secondary production, respiration) of the microbial communities that often control the fate of plant carbon and the immobilization or mineralization of detrital nutrients, such as nitrogen and phosphorus. This is particularly true for litter-associated fungal decomposers. As a consequence, much of our

knowledge about carbon and nutrient cycling in freshwater marshes and the quantitative contribution of various microbial groups is limited and awaits further study.

To date, most studies examining emergent plant decomposition in freshwater marshes have focused on mass loss rates of plant matter that had been prematurely harvested (living green) or manipulated (ground, oven-dried) before being artificially enclosed in litter bags at, or buried within, the wetland surface sediments (e.g., Poulnin 1984; Webster and Benfield 1986; Bayley and Mewhort 2004; Bedford 2005; Rothman and Bouchard 2007; Fennessy et al. 2008). This methodological approach continues today and is likely grounded in the perception that microbial decomposition of emergent plant matter in marshes occurs exclusively at or within the marsh sediments, where it is driven principally by aerobic and anaerobic bacterial communities (see Gutknecht et al. 2006; Mitsch and Gosselink 2007; Reddy and Delaune 2008; Kayranli et al. 2010). This perception is in sharp contrast with studies in freshwater stream systems, where aquatic hyphomycete fungi have been widely recognized as an important microbial assemblage that is central to litter decomposition and detrital food resources for inhabitant consumers (macroinvertebrates) (Bärlocher 1992; Gessner and Chauvet 1994; Weyers and Suberkropp 1996; Gessner and Chauvet 1997; Suberkropp 1997; Suberkropp 2001; Gulis and Suberkropp 2003; Methvin and Suberkropp 2003; Carter and Suberkropp 2004; Gulis et al. 2006; Gessner et al. 2007; Gulis et al. 2008; Chung and Suberkropp 2009; Findlay 2010; Suberkropp et al. 2010). As a result, the role and quantitative contribution of fungal decomposers in wetland biogeochemical processes has not been fully realized or explicitly included in current conceptual or quantitative models describing carbon and nutrient flow pathways (see Mitsch and Gosselink 2007; Reddy and Delaune 2008).

A large reason for this gap in knowledge is that former studies examining emergent plant decay in marshes have often overlooked a key phenologic attribute that is common to most emergent wetland plants (Newell 1993; Bärlocher 1997; Gulis et al. 2006; Kuehn 2008). In emergent macrophytes, the abscission and subsequent collapse of plant material to the sediments or overlying surface waters does not typically occur following plant senescence and death. As a result, standing-dead plant litter can accumulate in freshwater marshes (Asaeda et al. 2002; Christensen et al. 2009), where it begins initial microbial decomposition in an aerial standing-dead position (Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008). Seasonal estimates of *Juncus effusus* (soft rush) and *Typha angustifolia* (cattail) plant mass indicate that standing-dead litter can be a quantitatively significant pool of carbon and nutrients in both subtropical and temperate freshwater marshes, respectively (Kuehn 2008). For example, Kuehn (2008) reported that standing-dead litter of *J. effusus* in a subtropical marsh system ranged from 0.8 – 2.3 kg AFDM/m² over an annual period, which at times was nearly equivalent to the amount of aboveground living plant biomass (Wetzel and Howe 1999).

These findings indicate that natural decomposition of emergent plants in freshwater marshes consists of two distinct decay phases that are separated in space and time. There is an initial decay phase resulting from microbial processes that occur under aerial standing-dead conditions, followed by a second benthic decay phase resulting from microbial processes that occur under submerged or surface sediment conditions following the collapse of standing litter.

Standing-dead litter and fungal presence. Considerable evidence has accumulated for over a century (e.g., Saccardo 1898) that fungal decomposers

pervasively colonize and reproduce on and within both standing and collapsed litter of freshwater emergent macrophytes (Pugh and Mulder 1971; Apinis and Taligoola 1974; Apinis et al. 1975; Poon and Hyde 1998; Tsui and Hyde 2003; Gessner and van Ryckegem 2003; van Ryckegem and Verbeken 2005a; 2005b; 2005c; van Ryckegem et al. 2007). For example, Gessner and van Ryckegem's (2003) review of the literature indicated that >600 species of fungi have been recorded from the common reed, *Phragmites australis*. Furthermore, several investigators have observed distinct temporal and spatial shifts in fungal taxa associated with emergent plant litter substrates (leaves, stems) during decomposition (Poon and Hyde 1998; Van Ryckegem and Verbeken 2005a; 2005b; Van Ryckegem et al. 2007), implying that variable environmental conditions between the standing and submerged/sediment decay phases and/or intrinsic differences in litter substrate quality (e.g., lignin and nutrient concentrations) may influence the growth and decay performance of particular fungal species.

Fungal biomass and rates of fungal secondary production. Despite the evidence of litter accumulation in freshwater marshes and its pervasive colonization by fungi, our quantitative understanding of fungal functional processes remains limited. A major reason for this paucity of data can be attributed to a lack of reliable methods to accurately quantify biomass and production rates (growth rates and turnover) of fungal decomposers within decaying plant litter. As a result, our knowledge of fungal involvement in litter decay processes and their quantitative contribution to biogeochemical cycles has been understudied in most ecosystems (Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008; Gulis et al. 2009). However, growing evidence over the last several decades has revealed the usefulness of the fungal membrane sterol, ergosterol, in the quantification of living

fungus biomass in decaying plant litter and the technique for measuring *in situ* instantaneous growth rates of fungi from rates of [^{14}C]-acetate incorporation into ergosterol (Newell and Fallon 1991; Gessner and Newell 2002; Gessner 2005; Suberkropp and Gessner 2005; but see Mille-Lindblom et al. 2004).

Additional methods to assess fungal biomass from chitin, via glucosamine analysis, have also been described, primarily for terrestrial plant root samples (Ekblad and Nasholm 1996). Chitin, a polysaccharide consisting of *N*-acetyl-D-glucosamine, is a major component of fungal cell walls. Since chitin (glucosamine) remains in the fungal cell wall after death, it has been mentioned as a potential chemical indicator of combined living and dead fungal mass (Hicks and Newell 1984; Ekblad et al. 1998). The method appears promising for detection of fungal chitin in plant materials. However, this method has not been ground-tested or widely applied in the analysis of fungal chitin from decaying plant litter and thus awaits further development and testing before it can be used with confidence.

The use of the ergosterol to quantify fungal biomass in freshwater marshes has provided key evidence that fungal decomposers are a quantitatively important in the decomposition of emergent plant litter. Appreciable fungal biomass accumulation in both standing-dead and submerged plant litter has been observed in both subtropical and temperate freshwater marshes (Newell et al. 1995; Bärlocher and Biddiscombe 1996; Kuehn and Suberkropp 1998a, 1998b; Kuehn et al. 1999; Komínková et al. 2000; Kuehn et al. 2000; Gessner 2001; Findlay et al. 2002; Newell 2003; Verma et al. 2003; Welsch and Yavitt 2003; Buesing and Gessner 2006; Su et al. 2007; Kuehn et al. 2011; Kuehn et al. 2014). For example, Kuehn et al. (2011) recently reported that fungal biomass in

decaying *T. angustifolia* leaf blades reached concentrations as high as 10% of the total detrital weight.

To date, only a handful of studies have applied the ergosterol and ^{14}C -acetate incorporation methodology to estimate the growth and production, respectively, of fungal decomposers during plant litter decomposition in freshwater marshes (10 total studies - Newell et al. 1995; Komínková et al. 2000; Kuehn et al. 2000; Findlay et al. 2002; Newell 2003; Verma et al. 2003; Buesing and Gessner 2006; Su et al. 2007; Kuehn et al. 2011; Kuehn et al. 2014), and nearly all of these studies have been conducted in north temperate environments on decaying plant litter under submerged conditions. Furthermore, very few published studies have assessed the quantitative contribution of fungal decomposers to standing-dead litter decomposition (Gessner 2001; Kuehn et al. 2011), which were also conducted in north temperate environments. Recently, Kuehn et al. (2011) reported that significant transformation and decomposition of *T. angustifolia* litter occurred during the standing-dead phase (55% mass loss), and that a large portion of the plant carbon and nutrients were channeled into and through litter-associated fungal decomposers. Estimated cumulative fungal production during *Typha* decay totaled 123 mg C g⁻¹ initial detrital C, indicating that 22% of the *Typha* leaf carbon that was lost was transformed into fungal biomass. Furthermore, fungi associated with *Typha* litter also transformed and immobilized large amounts of detrital nutrients, with fungal biomass sequestering >50% of the total detrital nitrogen and phosphorus during leaf litter decay. Findings by Kuehn et al. (2011) demonstrated that fungal decomposers are likely important pathways of carbon and nutrient flow in freshwater marshes.

Rates of microbial respiration. If fungal decomposers are a significant colonizer of standing and collapsed plant litter, as previous studies have suggested, then the resulting metabolic activity (CO₂ evolution) observed from decaying litter may be largely attributable to the metabolic activities of litter inhabiting fungal decomposers (Kuehn and Suberkropp 1998a; Kuehn et al. 1998; Kuehn et al. 1999; Welsch and Yavitt 2003; Kuehn et al. 2004; but see Buesing and Gessner 2006). Studies conducted in subtropical and temperate freshwater marshes have established that microbial assemblages associated with standing-dead litter are capable of mineralizing plant litter carbon before its collapse to the sediments or surface waters. For example, Kuehn et al. (2004) examined the effects of environmental conditions on rates of microbial respiration (CO₂ evolution) from standing-dead litter (i.e., leaves, leaf-sheaths and culms) of the common reed, *P. australis*, in two temperate lake-littoral freshwater marshes in Switzerland. Under field conditions, rates of microbial respiration from standing litter exhibited a diel periodicity, with the highest rates occurring at night when water becomes available (dew formation) to litter inhabiting microbial assemblages. In contrast, microbial respiratory activities virtually ceased during the day as a result of increased microbial desiccation stress. Rates of microbial respiration (CO₂ evolution) from decaying *P. australis* leaves ranged from 5 to 223 µg CO₂-C/g AFDM/h and leaf sheaths from 10 to 155 µg CO₂-C/g AFDM/h over a daily period. Rates of microbial respiration from *P. australis* leaf sheaths were consistently lower (~25%) than those observed from decaying leaves, but were considerably higher than rates observed from decaying *P. australis* culms (Kuehn et al. 2004).

Differences in patterns and rates of microbial respiration observed among standing *P. australis* litter fractions (leaves vs. sheaths vs. culms) were reflected in differences in water absorption patterns, litter structural characteristics (lignocellulose), and the degree of fungal colonization. Maximum rates of respiration among litter fractions were positively correlated with litter associated fungal biomass (Kuehn et al. 2004). Similar correlations between rates of microbial respiration (CO₂ evolution) and litter-associated fungal biomass have also been reported from standing litter of *J. effusus* (Kuehn and Suberkropp 1998a) and a variety of other freshwater and salt-marsh emergent plant species (Newell 2003) in subtropical marshes. These studies illustrate that fungi inhabiting emergent plant litter are metabolically active and adapted (Kuehn et al. 1998) to the standing litter environment and can play a significant role in the mineralization of plant detritus in freshwater marshes.

Ecosystem-scale impacts of fungi in freshwater marshes. As discussed above, the few studies examining fungal biomass and rates of fungal production associated with decaying emergent plant litter have focused exclusively on quantifying changes during specific litter decay periods (i.e., from post plant senescence to *x* number of days). Emergent plant litter of known age and type (e.g., leaves, stems) were either placed in litter bags or tagged/sampled in a standing position and sampled through time (Newell et al. 1995; Kuehn et al. 1999; Kuehn et al. 2000; Komínková et al. 2000; Findlay et al. 2002; Verma et al. 2003; Newell 2003; Su et al. 2007; Kuehn et al. 2011). This approach has been useful in examining the dynamics of fungal decomposers in the transformation of plant litter carbon during various decomposition stages. However, to quantify the potential contribution of fungi or other microbial assemblages to ecosystem-scale carbon

and nutrient cycling in freshwater marshes it is necessary to have areal estimates of microbial biomass and production associated with naturally-occurring plant litter, which would include plant litter in various stages of decay (see Suberkropp 1997; Methvin and Suberkropp 2003; Carter and Suberkropp 2004; Suberkropp et al. 2010).

Currently, only three estimates of this kind are available for litter decomposing fungi in freshwater marsh ecosystems (Buesing and Gessner 2006; Kuehn 2008; Ohsowski 2008), and only one estimate has been reported for litter-associated fungi in salt-marsh ecosystems (Newell 2001a). Newell (2001a) was the first to estimate a rough annual areal fungal production associated with decaying plant litter in a subtropical salt-marsh ecosystem (Georgia). Production of fungal decomposers associated with standing-dead *Spartina alterniflora* litter totaled 230 gC/m²/y, indicating that roughly 40% of the annual cordgrass production was transformed into fungal biomass. Ohsowski (2008) estimated both annual bacterial and fungal production rates associated with standing *T. angustifolia* litter in a north temperate freshwater marsh in Michigan. Total annual fungal production associated with standing-dead *Typha* litter (leaves and shoots) was 43 gC/m²/y, which was significantly higher than corresponding annual estimates of bacterial production (7 gC/m²/y). These findings indicate that fungal biomass production may be a major pathway of ecosystem-level carbon flow during standing litter decomposition in emergent macrophytes.

What may be the potential implications of litter-associated microbial activities (CO₂ flux) for marsh carbon cycling at larger ecosystem scales? Based on microbial respiration rates and estimates of litter standing stocks (6 dates only), Kuehn and Suberkropp (1998a) provided a very rough daily carbon (CO₂) flux estimate of between

1.4 and 3.4 g C/m²/d for microbial assemblages inhabiting standing *J. effusus* litter in a subtropical freshwater marsh in Alabama. These estimates were equal to or exceeded benthic CO₂ flux estimates reported by Roden and Wetzel (1996) from the same subtropical wetland site, indicating that inhabitant fungal communities can potentially mineralize a significant fraction of the plant litter carbon. Carbon dioxide flux rates reported by Kuehn et al. (2004) for microbial communities inhabiting standing *P. australis* litter in a temperate freshwater marsh were lower (0.05-0.56 g C/m²/d), but still within the range of daily CO₂ flux estimates reported from marsh sediments in more northern climates (e.g., Scanlon and Moore 2000). Ohsowski (2008) also reported that microbial decomposers (bacteria and fungi) inhabiting standing litter could mineralize appreciable amounts of detrital carbon over an annual period. Estimates of total annual microbial respiratory flux from standing-dead *Typha* litter (leaves and shoots) was 57 gC/m²/y, suggesting that microbial mineralization of emergent plant litter during standing-litter decomposition may also be a significant source of CO₂ flux from freshwater marshes to the atmosphere.

Dissertation research objectives. To date, very little quantitative data is available concerning the role and contribution of fungal decomposers to plant litter decomposition (i.e., litter mass loss) in freshwater marsh ecosystems, particularly from subtropical climates. Furthermore, comparative ecosystem-scale estimates of carbon and nutrient flow through fungal decomposers are virtually non-existent for freshwater marshes. In view of the paucity of more comprehensive data, our current understanding of fungal involvement in carbon flow and nutrient cycling in freshwater marshes is still incomplete and awaits further examination. This dissertation describes research that examined the

dynamics of litter-associated fungal decomposers in two subtropical freshwater marshes (*T. domingensis* and a mixed *Cladium jamaicense/Juncus roemarianus*) over an annual period. Although largely descriptive in nature, this dissertation research pursued research questions that focus on quantifying the roles of litter-associated fungal decomposers in wetland carbon and nutrient cycling pathways. Specifically, this research focused on an examination of fungal contributions at two differing scales: quantification of fungal contributions to fine scale plant litter decay processes (Chapter III) and large-scale quantification of fungal contributions to entire ecosystem (per m²) carbon flow and nutrient cycling (Chapter IV). In addition, during this dissertation research I worked towards a refinement of analytical techniques used for the determination of chitin (glucosamine) from both soils and decaying plant litter (Chapter II). The overarching rationale for this dissertation research was derived from (1) prior research to date suggesting the importance of litter-associated fungal decomposers and (2) a more explicit consideration of litter-associated fungi and their quantitative contribution to litter decomposition and ecosystem-level carbon and nutrient cycling in subtropical freshwater emergent marshes.

CHAPTER II

EVALUATION AND APPLICATION OF A METHOD TO ASSESS FUNGAL CHITIN CONCENTRATIONS IN DECAYING PLANT LITTER AND SOIL VIA HPLC ANALYSIS OF CHITIN GLUCOSAMINE RESIDUES

Introduction

Filamentous fungal organisms are widely known to colonize decaying organic matter in both terrestrial and aquatic habitats (Bärlocher 1992; Webster and Weber 2007; Moore et al. 2011). As such, fungi, like bacteria play a central role in the decomposition and recycling of carbon and nutrients within ecosystems. In addition to these critical decomposer functions, many fungi also establish symbiotic associations with the roots of vascular plants. These mycorrhizal fungi form complex mycelial networks within plant roots and the surrounding extramatrical soil, where they serve as important mediators of carbon and nutrient exchange between the plants and the soil environment (Allen 1991; van der Heijden and Sanders 2003).

Despite the evidence indicating extensive fungal colonization of soils and decaying plant litter, our current quantitative understanding of fungal processes in ecosystems has lagged far behind other microbial groups, such as bacteria. Historically, comprehensive assessments of fungal processes have been hindered by the inability to quantify fungal biomass and rates of growth and production within field collected samples. The major impediment to such data has been the intimate pervasive association of fungi with the substrates they colonize, which conceals their filamentous somatic body (hyphae) and makes estimation of their biomass and growth rates extremely difficult (Gessner and Newell 2002).

However, compelling evidence has developed over the last several decades on the usefulness of the fungal sterol, ergosterol, as a biochemical index molecule for quantifying fungal biomass (Gessner and Newell 2002; Gessner 2005). Additional techniques have also been developed for measuring instantaneous growth rates of fungi via incorporation rates of [$1\text{-}^{14}\text{C}$]-acetate into ergosterol (Newell and Fallon 1991; Gessner and Newell 2002; Suberkropp and Gessner 2005). Ergosterol (ergosta-5,7,22-trien-3 β -ol) is largely a fungal-specific lipid of cell membranes (Weete et al. 2010) and its concentration within environmental samples is considered a promising indicator of living fungal biomass (Gessner and Newell 2002; Gessner 2005; but see Mille-Lindblom et al. 2004). Both of these methods have been increasingly used within a variety of aquatic and terrestrial habitats (e.g., Suberkropp 1997; Newell and Porter 2000; Bååth 2001; Wallander et al. 2001; Hendricks et al. 2006; Gulis et al. 2006; Sims et al. 2007; Gessner et al. 2007; Rousk and Bååth 2007; Suberkropp et al. 2010; Rousk and Bååth 2011; Wallander et al. 2011; Clemmensen et al. 2013; Wallander et al. 2013), which has produced important quantitative information concerning the functional role of fungal organisms in carbon and nutrient cycling pathways.

In addition to ergosterol, fungal colonization and biomass has also been estimated from chitin concentrations. Chitin, a linear polysaccharide consisting of N-acetyl-D-glucosamine subunits, is the principle component of fungal cell walls (Feofilova 2010). In comparison to ergosterol analysis, the analytical procedure for determining chitin is inherently more complicated, which involves the initial extraction and then digestion of the chitin polymer followed by the analysis of resulting D-glucosamine residues. Fungal chitin is considered a recalcitrant molecule (but see Fernandez and Koide 2012), which

can remain after the death of fungal hyphae. As a consequence, it has been used in combination with ergosterol as potential biochemical indicator molecule of both living and dead fungal mass (Hicks and Newell 1984; Ekblad et al. 1998).

Earlier, Ekblad and Näsholm (1996) developed a high performance liquid chromatography (HPLC) method to quantify chitin concentrations in plant roots colonized by ectomycorrhizal fungi. This analytical procedure involved the HPLC measurement of fluorescent glucosamine derivatives following alkaline extraction and high-temperature acid digestion of the chitin polymer in fungal colonized root tissues. As noted by Ekblad and Näsholm (1996), a potential problem underlying the method is the presence of residual plant and fungal degradation products from the extraction and digestion procedure, which could interfere with the fluorescence-based HPLC detection and analysis. Furthermore, additional studies have documented that the acid concentration, incubation temperature, and digestion time can have a significant influence on the release of glucosamine residues from the chitin polymer (Chen and Johnson 1983; Tolaimate 2003; Zhu et al. 2005), which could affect the accurate quantification of chitin concentrations within collected samples.

Although a promising technique for determining fungal chitin, the Ekblad and Näsholm (1996) method has not been sufficiently ground-tested or widely applied to other fungal colonized substrates, such as soils or decaying plant litter. As a consequence, the method awaits further refinement and development before it can be used with confidence. The major objective in the present study was to investigate and adapt the Ekblad and Näsholm (1996) method for the analysis of fungal chitin in soils and decaying plant litter.

Materials and Methods

Determination of acid and temperature digestion conditions. Overall, the Ekblad and Näsholm (1996) method for chitin determination involves three broad procedures that include: (1) Initial alkaline extractions (0.2N NaOH) and distilled water washes to deproteinate samples and partially deacetylate the sample chitin polymer to chitosan, (2) High temperature acid digestion (HCL) of sample chitosan into individual D-glucosamine residues, and (3) Fluorescent derivatization of glucosamine residues with 9-fluorenylmethylchloroformate (FMOC-Cl) and subsequent analyses glucosamine derivatives via HPLC.

To test the applicability of the Ekblad and Näsholm (1996) method for soils and decaying plant litter, I first examined the effects of temperature, acid concentration, and incubation time on the digestion and release of glucosamine residues in soil samples containing known amounts of chitin. During this experiment, ~1.0 mg of raw chitin from shrimp shells (Sigma-Aldrich, catalogue number C-7170) was mixed with 2.5 g of muffled sandy soil (4h @ 500°C) in sterile 50 ml polypropylene centrifuge tubes with plug-seal caps (VWR catalogue #89039-662). To remove soluble carbohydrate and proteinaceous material, samples were initially extracted following Ekblad and Näsholm (1996) protocols in 0.2M NaOH (10 ml total volume) for 6 h at room temperature (~20°C). After 6 h, samples were briefly vortexed, centrifuged for 10 min. @ 2000 rpm, and the supernatants carefully removed by vacuum aspiration. Ten ml of fresh 0.2M NaOH was then added to samples, vortexed briefly, and sample extraction continued by placing tubes in a dry-bath heating block at 100°C for 17 h. After 17 h, samples were removed, cooled to room temperature, vortexed, centrifuged, and supernatants aspirated

as above. Samples were then washed at least 4+ times with 20 ml of distilled water using the same procedure above (i.e., vortexing, centrifuging, and aspirating supernatants) until the color of resulting sample supernatants were clear.

The resulting chitosan in soil samples were then hydrolyzed using HCL acid into individual glucosamine residues. To determine optimal conditions for acid hydrolysis of sample chitin, I employed a three level assay experiment to test the effect of acid concentration (6, 8, 10M HCL), incubation temperature (80, 90, 100, 110°C), and time (4, 6, 8, 12, 24 h) on chitin digestion and release of glucosamine residues. During these experiments, three replicate chitin-soil mixtures (~1mg chitin) were digested at each acid/temperature/time combination in a dry-bath heating block. Following digestion, glucosamine residues in acid hydrolyzates were converted to 9-fluorenylmethylchloroformate (FMOC-Cl) derivatives following modified procedures described by both Ekblad and Näsholm (1996) and Zhu et al. (2005). Twenty µl aliquots of hydrolyzate was placed into a 1.5 ml screw-capped microcentrifuge tube and neutralized with ~19 µl of 8M NaOH to a pH ~7. Previously, Zhu et al. (2005) reported that pH 7 was the optimal pH for 9-fluorenylmethylchloroformate (FMOC-Cl) derivatization of glucosamine. To this mixture, 211 µl of 0.5M sodium borate buffer (pH=7) was added and briefly vortexed. Glucosamine residues were then derivatized by the addition of 250 µl of 15mM 9-fluorenylmethylchloroformate solution (in HPLC grade acetonitrile) and allowed to incubate in darkness for 30 minutes at room temperatures (~20 °C). After derivatization, the FMOC-glucosamine samples were transferred to Shimadzu HPLC autosample vials (1.5 ml), mixed with an additional 500 µl of borate buffer, and stored in the refrigerator at 4 °C until analyzed.

The method described by Ekblad and Näsholm (1996) for the HPLC separation and detection of Fmoc-glucosamine derivatives involved a multiple mobile phase gradient that was rather complex and long (40 minutes). As a consequence, I sought technical advice from HPLC chemists at Grace Davison Discovery Sciences (formerly Alltech Scientific) on an alternative separation technique. Based on this advice, I adopted a more simplified binary mobile phase gradient for the HPLC analysis of fluorescent glucosamine derivatives, which was very similar to HPLC running conditions described earlier by Zhu et al. (2005). The separation and quantification of Fmoc-glucosamine derivatives was performed using a Shimadzu High Pressure Liquid Chromatography (HPLC) system equipped with a LichroSpher 100 reverse phase C-18 column. The mobile phases were (A) 5 mM ammonium formate (pH=4.5) and (B) HPLC-grade acetonitrile at a total flow rate of 1.0 ml/min. A binary gradient was performed from 30% to 100% mobile phase (B) acetonitrile over 13 minutes. The column was then regenerated with 100% acetonitrile for 4 minutes, before returning to initial running conditions (total run time 21 minutes). Fmoc-glucosamine was detected using a Shimadzu (RF-10AXL) fluorescence detector (excitation 260nm, emission 330nm) and was identified based on comparison with known D-glucosamine standards (range 0 – 50 µg/ml, Sigma-Aldrich) that were simultaneously derivatized along with acid hydrolyzed samples.

Test of chitin assay procedure. Based on data resulting from acid and temperature digestion experiments (see results below), I developed an extraction, digestion, and derivatization protocol to examine the effectiveness of assay procedure for analyzing chitin in soil and plant material over a range of chitin concentrations. A simplified flow diagram outlining the developed protocol is presented in Figures 1 and 2. Samples of

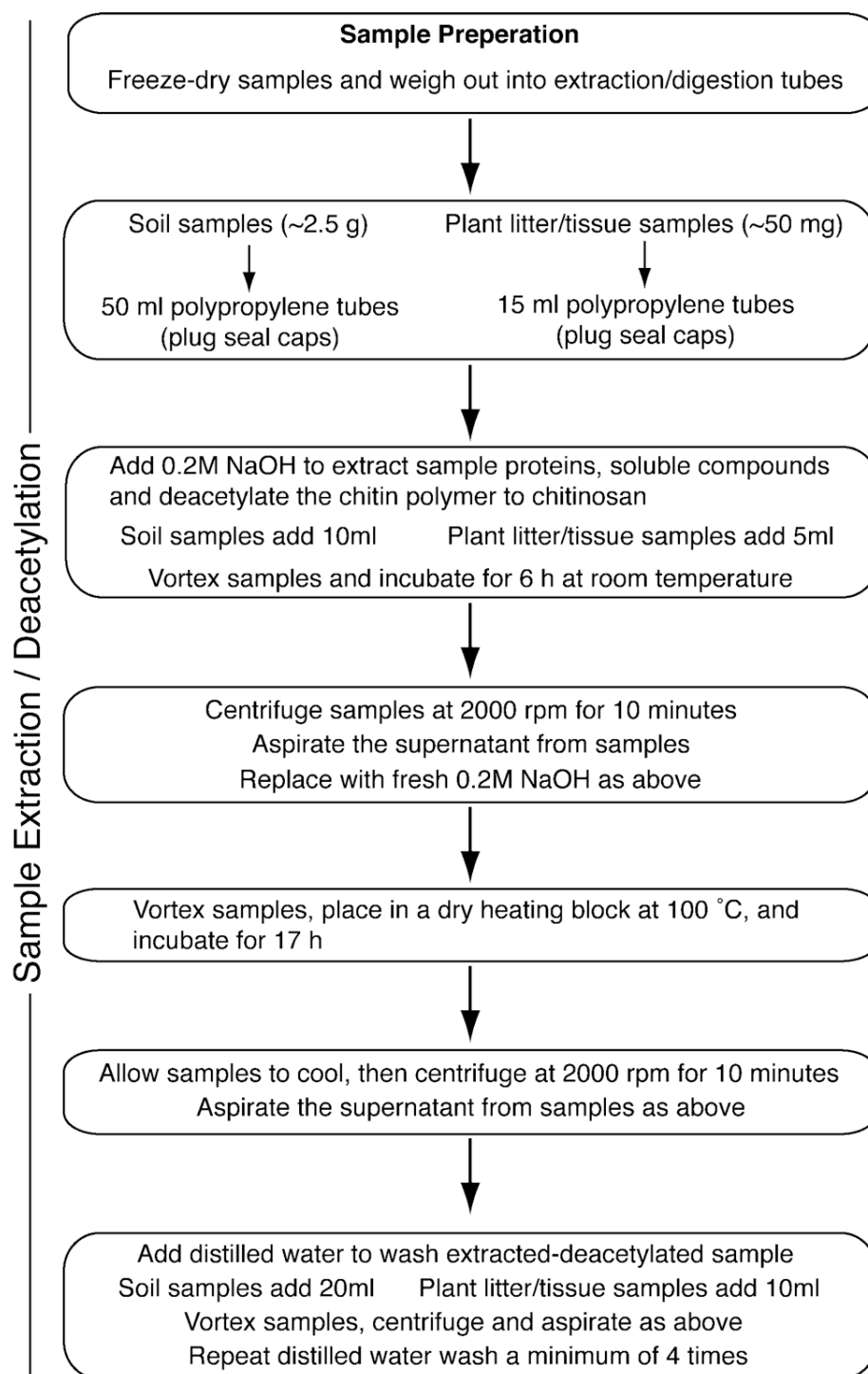


Figure 1. Proposed procedure for extracting and deacetylating chitin in soil and plant litter samples.

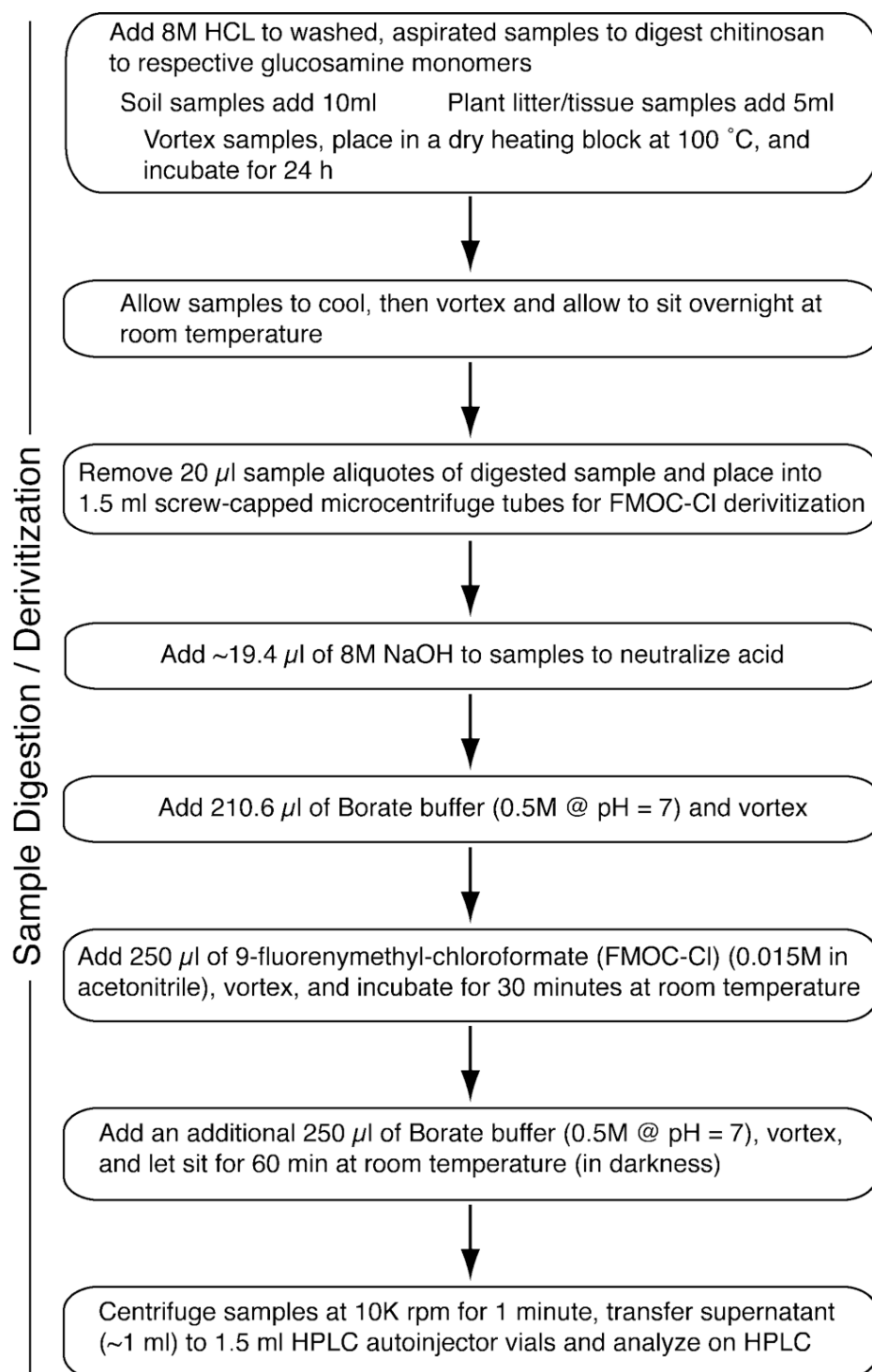


Figure 2. Proposed procedure for acid digestion of chitin and FMOCl-Cl derivatization of resulting glucosamine residues in soil and plant litter samples.

chitin-soil and chitin-plant mixtures containing a range of known chitin concentrations (0 – 3 mg, Sigma-Aldrich as above) were extracted, digested, derivitized, and analyzed via HPLC. For chitin-soil mixtures, a total of 8 separate assay experiments were conducted to examine the reproducibility of the assay procedure. Six separate experiments were conducted using chitin-soil mixtures containing a high range of added chitin (0, ~1, ~2, and ~3 mg of chitin, n= 3-4 each concentration). Two additional experiments were also conducted using a lower concentration range of chitin (i.e., between 0 - 1 mg) to assess the method's effectiveness for detecting low-level chitin concentrations (10 concentrations, n=10 each). During these experiments, chitin was mixed with 2.5 g of muffled soil (as above) in sterile 50 ml polypropylene centrifuge tubes, and samples were extracted, digested, and derivitized following the protocols outlined in Figures 1 and 2.

Likewise, for chitin-plant mixtures a total of 3 separate assay experiments were conducted (2 – high range and 1 – low range chitin, as above) to assess the methods effectiveness and limit to analyze chitin in plant material. During these experiments, chitin was mixed with ~50 mg of ground (40 mesh – Wiley mill, ~425 μ m particle size) of living green *Typha* leaves in sterile 15 ml polypropylene centrifuge tubes with plug-seal caps. Samples were extracted, washed, and digested using the same procedure outlined above, except that the volume of 0.2M NaOH, distilled water, and 8M HCL added was exactly half of that used for soil samples (see Figures 1 and 2). The FMOC-Cl fluorescent derivatization and HPLC analysis of glucosamine residues in sample digests followed the same protocols as described above.

Results and Discussion

In the present study, the HPLC separation and analysis of FMOC derivatized glucosamine standards revealed the co-occurrence of two peaks, with retention times of 6.1 and 6.4 minutes, respectively. Resulting chromatograms of hydrolyzed chitin standards within chitin-soil and chitin-plant mixture samples revealed a similar chromatographic pattern, with two peaks being observed at the same retention times as corresponding glucosamine standards. Standard curves of D-glucosamine standards simultaneously derivatized along with chitin acid hydrolyzates during each experiment displayed a linear response over the range of glucosamine concentrations examined (0-50 $\mu\text{g/ml}$), with consistent slopes (coefficient of variation $<7\%$) and resulting r^2 values which were all >0.99 . Figure 3 shows the collective relationship between the concentration of standard glucosamine and the resulting total fluorescence peak area (mean \pm SD) observed following FMOC-CL derivatization and HPLC analysis.

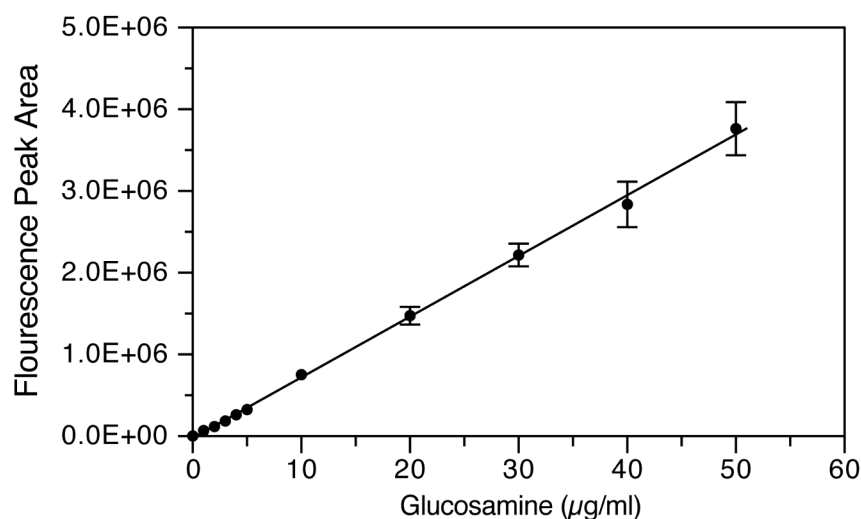


Figure 3. Relationship between D-glucosamine standards and HPLC fluorescence peak area (mean \pm 1SD) following FMOC-CL derivatization and HPLC analysis.

Similar chromatographic patterns have been reported by Ekblad and Näsholm (1996) and Zhu et al. (2005) for both derivitized D-glucosamine standards and glucosamine residues released following acid hydrolysis of chitin. During their studies, both derivitized D-glucosamine standards and chitin acid hydrolyzates showed the presence of two dominant peaks during HPLC analysis, which established that the peak analytes were not the direct result of HCl acid hydrolysis but instead resulted from the derivatization of free glucosamine residues by FMOC-CL. Furthermore, the consistent observation of dual glucosamine peaks in the present study lend support to the findings of Ekblad and Näsholm (1996) and Zhu et al. (2005), who speculated that the presence of two peaks in HPLC chromatograms was likely due to the presence of both α and β anomeric forms of glucosamine (see also Daiz et al. 1996).

Initial experiments examining the conditions for acid hydrolysis of chitin indicated that the HCL concentration, temperature and heating time all had a significant effect on chitin digestion and the release of glucosamine residues (Figure 4, Table 1). Similar findings were also observed by Zhu et al. (2005), which reported that both the concentration of HCl and digestion temperatures were important variables influencing the hydrolysis of the chitin polymer. In the present study, the recovery of FMOC-glucosamine during chitin digestion experiments revealed a maximum recovery (fluorescence peak area) using an HCl acid concentration of 8M and a digestion temperature and heating time of 100°C and 24 h, respectively (Figure 4), which contrast slightly with acid hydrolysis conditions used earlier by Ekblad and Näsholm (1996) and Zhu et al. (2005).

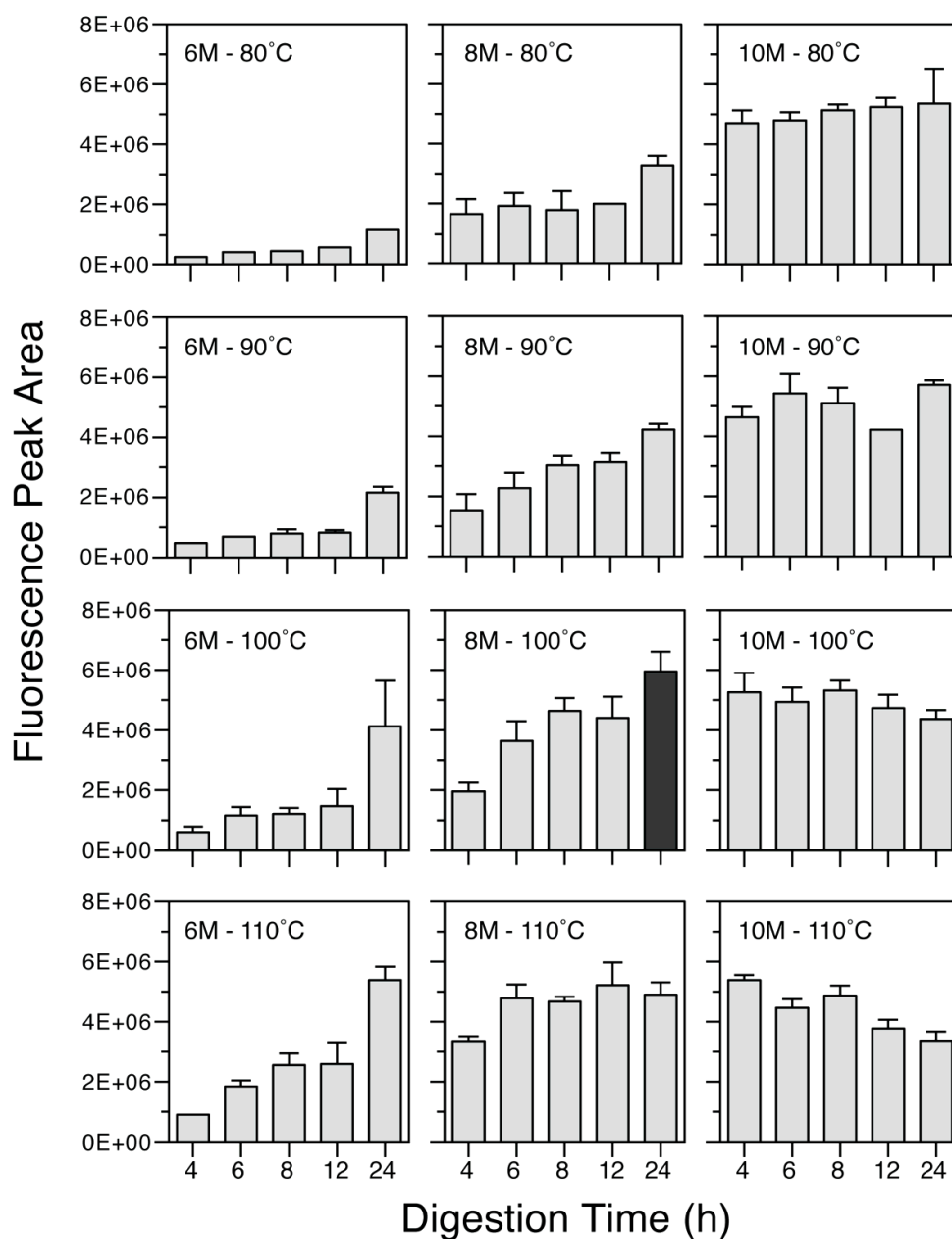


Figure 4. Effect of HCl concentration, incubation temperature, and time on chitin digestion in experimental soil samples containing known amounts of raw chitin. Fluorescence peak areas (mean \pm 1SD) indicate the concentration of FMOc derivitized D-glucosamine residues in resulting chitin acid hydrolyzates following digestion procedures. Optimal conditions for chitin digestion is indicated by the black-shaded bar.

Table 1

ANOVA summary indicating HCl acid concentration, temperature, and time effects on chitin digestion in experimental soil samples.

Source	df	F-Ratio	p-Value
Acid concentration	2	769.03	<0.001
Temperature	3	60.26	<0.001
Time	4	74.94	<0.001
Acid x Temperature	6	24.90	<0.001
Acid x Time	8	42.50	<0.001
Temperature x Time	12	1.86	<0.05
Acid x Temperature x Time	24	6.54	<0.001
Error	118		

For example, Ekblad and Näsholm (1996) examined the effect of time (1-26 h) on chitin digestion in fungal-plant root samples using a uniform acid concentration of 6M HCL and a digestion temperature of 100 °C. Glucosamine from chitin digest showed a maximum recovery after a 7 h of digestion. In a more detailed study, Zhu et al. (2005) examined 3 HCl acid concentrations (6, 8, 10M), 3 temperatures (100, 110, 120°C), and 3 heating times (2, 4, 6 h) on the digestion and release of glucosamine from pure chitin.

Results of their study indicated that the optimum conditions for acid hydrolysis of chitin were achieved at 110°C with 8M HCL. The influence of heating time was noted, but was considered less important than the acid concentration and incubation temperature.

Collectively, the results obtained in these former studies and the present investigation highlight that chitin hydrolysis conditions must be carefully evaluated, as the optimal acid digestions conditions might differ among the specific substrates being analyzed.

Like standard glucosamine assays, acid digestion and subsequent FMOC-CL derivatization of glucosamine residues in chitin-soil and chitin-plant samples also displayed a linear response over the range of chitin concentrations examined (0-3 mg), indicating that the modified methodological approach developed in this study was effective in estimating chitin (glucosamine) concentrations in both soil and plant litter substrates. Furthermore, separation and detection of chitin in chitin-plant mixtures indicated minimal interference by residual plant or other acid degradation, which confirms that the initial NaOH extraction and DI water wash procedures described by Ekblad and Näsholm (1996), and used in the present study, were sufficient in removing any chemical products that could interfere with the HPLC detection of derivatized glucosamine residues.

Figure 5A and B illustrates the relationship between the concentration of added chitin and the derivitized glucosamine fluorescence peak area (mean±SD) for both chitin-soil and chitin-plant mixtures in each of the experimental digestions, respectively. The results of regression analysis indicated a significant relationship for both chitin-soil ($r^2 = 0.98$, $p < 0.001$) and chitin-plant assays ($r^2 = 0.98$, $p < 0.001$), indicating that the recovery and analysis of FMOC-glucosamine residues in chitin acid digest are a promising

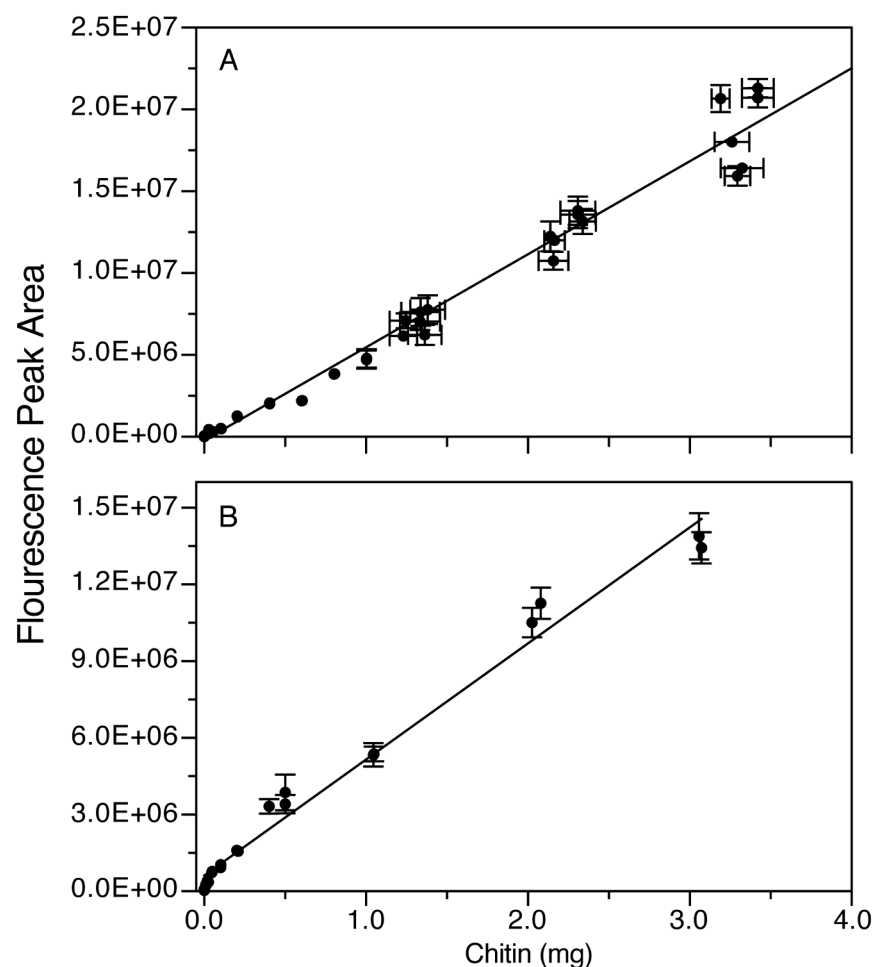


Figure 5. Relationship HPLC fluorescence peak area (mean \pm SD) and chitin concentrations in known (A) chitin-soil and (B) chitin-litter mixtures. Fluorescence peak areas (mean \pm 1SD) indicate the concentration of FMOC derivitized D-glucosamine residues in resulting chitin acid hydrolyzates following digestion procedures.

indicator for determining chitin in both soil and plant litter samples. However, although promising for detecting chitin, comparison of FMOC-glucosamine data from chitin-soil and chitin-plant acid digests do show some significant fluorescence peak area differences between the plant and soil substrates. Furthermore, there was noted variability in the fluorescence peak area detected between individual digestion assays, particularly those at higher added chitin concentrations (e.g., \sim 3 mg chitin, see Figure 5A). These findings

highlight that it is critical to include a series of both known glucosamine and chitin standards during each sample assay procedure.

The present method has been now used in two field-based studies that have sought to assess the dynamics of fungal decomposers during standing-dead litter decay of *T. angustifolia* (Kuehn et al. 2011) and *T. domingensis* (Chapter III of this dissertation).

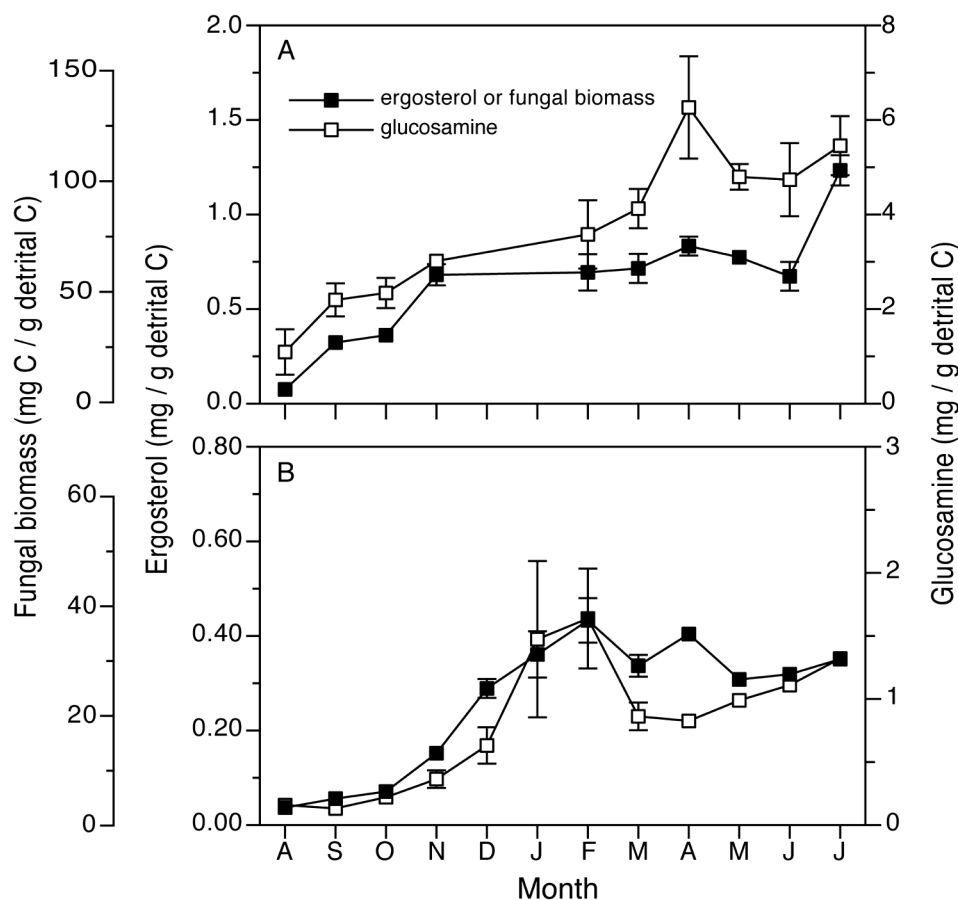


Figure 6. Dynamics of ergosterol and glucosamine concentrations in standing-dead *T. angustifolia* leaves in Michigan (A) and standing-dead *T. domingensis* leaves in Alabama (B). Glucosamine concentrations were determined using the modified method described in this chapter. Estimated fungal biomass is also indicated. Symbols indicate the mean \pm 1SE.

Specifically, patterns of both chitin (glucosamine) and ergosterol concentrations associated with *Typha* litter were examined during plant senescence and early standing litter decomposition. Results obtained from both studies show that chitin concentrations

associated with decaying *Typha* leaves followed a very similar pattern as ergosterol concentrations (Figure 6), with both glucosamine and ergosterol concentrations being significantly related to each other ($r^2 = 0.911$, $p < 0.001$)(Figure 7).

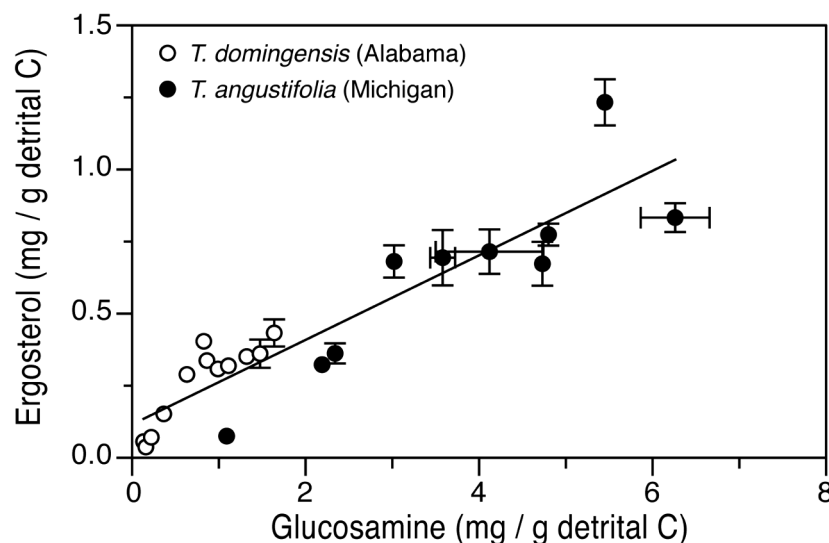


Figure 7. Relationship between ergosterol and glucosamine concentrations in standing-dead leaf litter of *T. angustifolia* (Michigan) and *T. domingensis* (Alabama). Symbols indicate the mean \pm 1 SE

These consistent findings in two independent studies provide compelling evidence that chitin determination in plant litter, and possibly soil, could be used as a potential biochemical index marker for the quantifying fungal mass (i.e., living and dead) associated within decaying plant litter. However, unlike ergosterol, very few studies have determined conversion factors directly relating chitin (glucosamine) concentrations to fungal mass (e.g., Hicks and Newell 1984; Ekblad et al. 1998). As a consequence, more pure culture studies are needed of a wide variety of fungal species in order to establish appropriate and realistic conversion factors.

CHAPTER III

FUNGAL CONTRIBUTIONS TO CARBON FLOW AND NUTRIENT CYCLING
FROM STANDING-DEAD *TYPHA DOMINGENSIS* LEAF LITTER IN A
SUBTROPICAL FRESHWATER MARSH

Introduction

Freshwater marshes are characterized by the prolific growth of emergent vascular plants, and as such are widely considered as among the most productive ecosystems on earth. Emergent vascular plants, such *Juncus*, *Cladium*, and *Typha*, are common in many freshwater marshes, and often comprise a significant portion of the organic matter produced within these ecosystems (Kvêt and Westlake 1998; Wetzel 2006; Mitsch and Gosselink 2007). These plants exhibit extremely high rates of primary production, with annual aboveground plant production alone often exceeding 1000 gC/m²/y. Furthermore, because of their prolific growth and concomitant nutrient demands, emergent plants also absorb and sequester large amounts of nutrients, such as nitrogen and phosphorus, and thus represent a quantitatively important reservoir pool of nutrients (Mitsch and Gosselink 2007; Reddy and Delaune 2008).

As in many ecosystems (Moore et al. 2004; Hagen et al. 2012), most of the plant biomass produced in freshwater marshes eventually enters the detrital pool following plant senescence and death, where microbial decomposers and detritus-feeding animals play an important role in its breakdown and mineralization. During plant decomposition, carbon and nutrients sequestered in plant tissues (e.g., lignocellulose) are released from detrital organic matter through the growth and decay activities of litter inhabiting microbial decomposers. Microbial growth and biomass accumulation within decaying

plant litter in turn serves as an important food resource for detritus-feeding consumers (e.g., Kulesza and Holomuzki 2006), which through their feeding activities help facilitate the further breakdown of plant litter. As a consequence, because large quantities of carbon and nutrients are bound in plant detritus, many characteristics related to marsh carbon and nutrient cycling are coupled with, and influenced by, the decay activities of litter-associated microbial decomposers. Determining the fate of plant material and the microbial processes involved during litter decay is therefore a key component to our quantitative understanding of energy flow and elemental cycling within these ecosystems.

Important details to consider when examining the decomposition of emergent vascular plants is both the spatial and temporal conditions under which plant material naturally decomposes. In many emergent macrophytes, the abscission and collapse of plant organs (e.g., leaves, culms) to the sediments or overlying surface waters does not typically occur after plant senescence and death. As a result, large amounts of standing-dead plant litter typically accumulate in freshwater marshes (e.g., Asaeda et al. 2002; Christensen et al. 2009), where it undergoes initial microbial decay in an aerial standing position (Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008; Gulis et al. 2009). Prior studies have demonstrated that this emergent standing litter is pervasively colonized by fungal decomposers (e.g., Gessner and van Ryckegem 2003), which are adapted to harsh environmental conditions present in the aerial standing litter environment (Kuehn et al. 1998).

Despite evidence of standing litter accumulation in freshwater marshes and extensive fungal colonization, much of our current quantitative understanding of fungal functional processes in these systems is limited. To date, only a few studies have

quantified rates fungal secondary production during standing (Newell et al. 1995; Findlay et al. 2002; Verma et al. 2003; Newell 2003; Kuehn et al. 2011) or collapsed litter decomposition (Sinsabaugh and Findlay 1995; Newell et al. 1995; Komínková et al. 2000; Kuehn et al. 2000; Findlay et al. 2002; Verma et al. 2003; Buesing and Gessner 2006; Su et al. 2007; Kuehn et al. 2014). As a result, fungal mediated carbon and nutrient flow processes within standing and collapsed litter compartments of freshwater marshes have not been recognized as a potential pathway in existing models of wetland biogeochemical cycles (Mitsch and Gosselink 2007; Reddy and Delaune 2008; Kayranli et al. 2010; but see Newell and Porter 2000).

Recently, Kuehn et al. (2011) examined the contribution of fungal decomposers to standing-dead leaf decomposition of *T. angustifolia* in a north temperate lake-littoral marsh in Michigan. Significant losses in *Typha* leaf carbon (~ 55%) occurred during standing litter decay, supporting prior research from both salt-marsh (Newell and Porter 2000) and freshwater marsh ecosystems (Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008) that appreciable carbon mass loss of emergent plant matter can occur in the standing-dead position. Corresponding estimates of fungal biomass and production rates in decaying litter indicated substantial growth and accrual of fungal biomass during *Typha* decomposition, establishing that fungal decomposers are effective in acquiring and assimilating plant litter carbon and nutrients (N and P) in the aerial standing litter phase. These findings provided compelling evidence that carbon flow through fungi may be an important yet unrecognized biogeochemical pathway in freshwater marshes.

Currently, the single production estimate reported by Kuehn et al. (2011) is the only detailed published report attempting to assess the quantitative contribution of fungal

decomposers to standing litter decomposition in freshwater marshes, which was conducted in a north temperate climate. Similar to Kuehn et al. (2011), the present study was conducted to examine patterns of litter mass loss and fungal dynamics associated with standing-dead emergent plant litter in a subtropical marsh climate. Seasonal biomass and production rates of fungal decomposers were estimated during early standing litter decomposition of *Typha domingensis* leaves in order to assess the contribution of fungal decomposers to carbon flow processes during the standing litter decay. Likewise, I also examined the growth and biomass dynamics of fungal decomposers relative to changes in plant litter nitrogen and phosphorus in order to examine their importance to nutrient cycling. Overall I predicted that rates of standing litter decay and corresponding fungal contributions to carbon and nutrient cycling in standing litter would be greater in a subtropical freshwater marsh as a result of more conducive environmental conditions for fungal growth (e.g., higher temperatures, relative humidity and longer growing season).

Study Site

This study was conducted in a small freshwater emergent *T. domingensis* marsh located in the Weeks Bay National Estuarine Research Reserve (NERR) near Magnolia Springs, Alabama, USA (N30°25.22, W87°49.36). The site is situated within an extensive marsh complex, which includes other marshes that are dominated by *C. jamaicense* and *J. roemerianus*. These marshes are separated from each other and the mainland by man-made canals. Collectively, this marsh complex is located near the mouth of the Fish River as it enters into the northern portion of Weeks Bay. Characteristics of Fish River surface waters that flow into the marsh complex are summarized in Table 3 of Chapter IV.

Materials and Methods

Field sampling procedures. Sampling and analysis of aboveground *T. domingensis* leaves for mass loss patterns, nutrient concentrations, and associated fungal biomass and production followed procedures previously described by Kuehn et al. (2011). Standing *T. domingensis* leaves (2008 cohort) were collected monthly from August 2008 to July 2009 in 6 randomly selected locations along a 50m transect extending through the *Typha* stand. During each collection period 1 leaf was randomly collected from 2 separate *Typha* shoots in each of the 6 locations to estimate losses in area-specific leaf carbon mass (see below). One additional *Typha* leaf was also randomly collected to determine litter-associated fungal biomass, rates of fungal growth and production, and rates of microbial respiration (see below). Collected samples were placed individually in clean zip-lock bags, placed on ice in a cooler, and immediately returned to the laboratory and processed.

Air temperatures and relative humidity were continuously recorded at 15 minute intervals throughout the entire study period using two Onset Hobo H8 Pro series data loggers (Figure 8), which were placed at mid-canopy height directly within the *Typha* stand. Daily precipitation data was also recorded via a permanent meteorological data station located near the *Typha* stand within the Weeks Bay reserve (Figure 8).

Leaf litter mass loss pattern and nutrient concentrations. Losses in *T. domingensis* leaf carbon were determined from losses in area-specific leaf mass following protocols described by Gessner (2001) and Kuehn et al. (2011). Upon return to the laboratory collected leaves were scanned using a flatbed scanner and corresponding picture files analyzed using the software program Canvas (Macintosh version 3.5.5) to

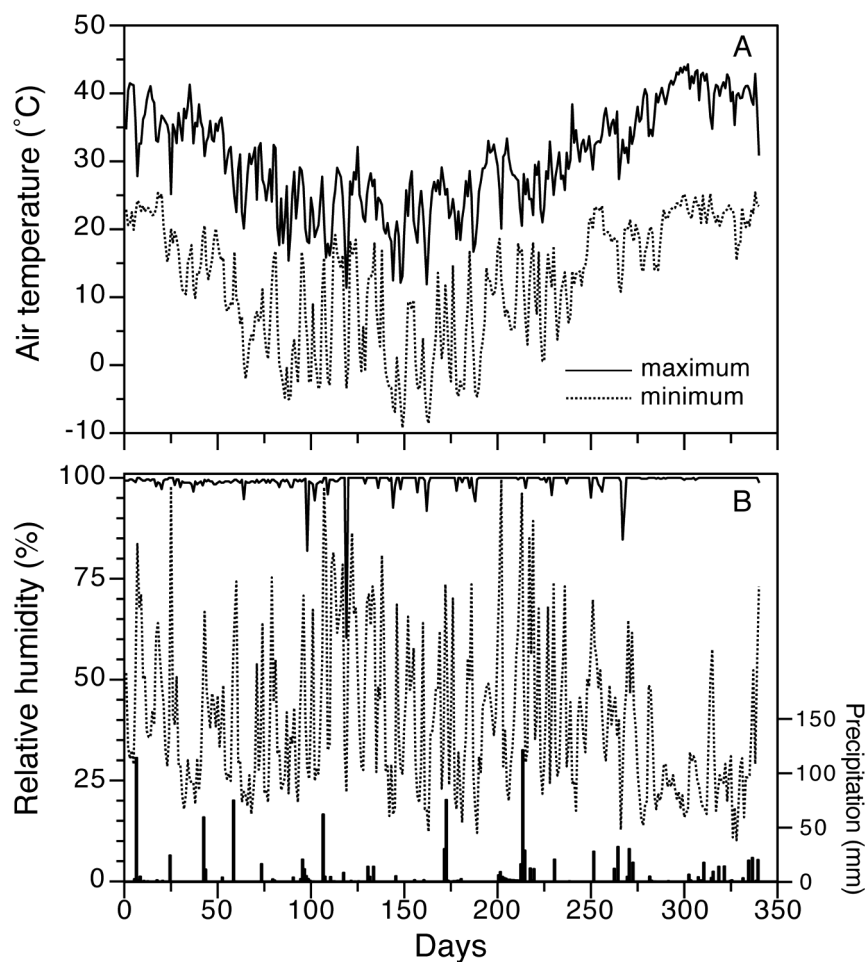


Figure 8. Changes in maximum (solid line) and minimum (dashed line) (A) air temperature, and (B) relative humidity and daily total precipitation (dark vertical bars) in the *T. domingensis* marsh stand during the annual study period.

determine total leaf surface areas. After scanning, leaf samples were placed back in the zip-lock collection bag and stored in the freezer (-20 °C) until analyzed. Frozen leaves were later lyophilized to dryness, weighed, and ground with a Wiley mill through a 40-mesh screen. Subsamples of ground plant material were subsequently used to determine plant litter carbon, nitrogen, phosphorus, and glucosamine concentrations (see below).

Initial area-specific leaf carbon mass of *T. domingensis* leaves were determined by dividing the leaf carbon mass at the initial sampling date when leaves were still living

(August 2008) by the corresponding scanned leaf area. Afterwards, the percent of leaf carbon mass remaining during later sampling dates was determined as changes in area-specific leaf carbon mass relative to the initial mean leaf area-specific carbon mass observed in August. The decay rate of *Typha* leaves ($-k$) over the study period was calculated using a nonlinear negative exponential regression model ($N_t = N_0 e^{-k(t)}$), where N_t is the percent of area-specific leaf carbon mass remaining at time (t) in days, N_0 is the estimated initial area-specific leaf carbon mass, and e is the base of the natural logarithm. *Typha* leaf carbon and nitrogen concentrations were determined using a Costech 4010 elemental combustion analyzer. Litter phosphorus concentrations were determined using a SEAL AA3 Flow Injection Nutrient Analyzer (molybdate-ascorbic acid method) following combustion (500°C) and hot HCl extraction of ground litter subsamples.

Fungal biomass and rates of fungal production. The living biomass and instantaneous growth rates of fungi associated with collected *Typha* leaves were estimated from litter-associated ergosterol concentrations and rates of [$1\text{-}^{14}\text{C}$]-acetate incorporation into ergosterol, respectively (Gessner 2005; Suberkropp and Gessner 2005). Two leaf sections (~2 cm) were cut from collected leaves and incubated for 4-5 h under dark conditions at 15 °C in clean, sterile glass scintillation vials containing 4 ml of filtered (0.22 µm membrane filter) marsh water and 5 mM Na[$1\text{-}^{14}\text{C}$]-acetate (final specific activity = 48 MBq/mmol). A killed control sample containing two *Typha* leaf sections and formalin (2% v/v final concentration) was also incubated to correct for any non-biological incorporation of ^{14}C radiolabel. Incorporation of [$1\text{-}^{14}\text{C}$]-acetate was stopped by placing vials on ice and immediately filtering the contents through a 1.2 µm

glass fiber filter. Sample leaf pieces and filters were washed twice with 4 ml of filtered marsh water, placed back into glass scintillation incubation vials, and stored frozen at -20 °C until analyzed. Later, frozen samples were lyophilized to dryness, weighed, and litter ergosterol extracted and quantified by High Pressure Liquid Chromatography (see Kuehn et al. 2011). Injected ergosterol fractions eluting from the HPLC were collected in 20 ml glass scintillation vials using an Advantec SF-3120 fraction collector, mixed with 10 ml of Ecolume scintillation fluid (MP Biomedical), and radioactivity determined using a Beckman LS6500 Scintillation Counter. Sample radioactivity was corrected for quenching and background radioactivity observed in corresponding killed controls. Litter associated ergosterol concentrations and radioactivity in ergosterol fractions were determined as the average of two HPLC injections per sample extract.

Estimates of living fungal biomass in leaf litter samples were inferred from ergosterol concentrations using a conversion factor of 5 µg ergosterol / mg fungal dry mass, and 43% carbon in fungal dry mass. Estimates of hourly fungal growth rates (μ , % / h), based on rates of [1-¹⁴C]-acetate incorporation into ergosterol, were calculated using a conversion factor of 12.6 µg fungal biomass synthesized / nmole acetate incorporated (Gessner and Newell 2002). Corresponding hourly rates of fungal production were determined by multiplying the fungal growth rate by the fungal biomass concentration.

Glucosamine concentrations from litter-associated chitin were also quantified as another potential bio-indicator molecule of total (living + dead) fungal mass. A detailed protocol describing the extraction, digestion and quantification of chitin as glucosamine is outlined in Chapter II of this dissertation. Briefly, samples were extracted and digested using a modified procedure originally described by Ekblad and Näsholm (1996).

Subsamples (~20 mg) of lyophilized, ground plant litter was first extracted in 0.2M NaOH and centrifuged litter pellets subsequently washed with distilled water to deproteinate samples and convert sample chitin to chitosan. Sample chitosan was then digested in 8M HCL, which hydrolyzed chitosan into individual glucosamine residues. Aliquots of acid hydrolyzates containing glucosamine residues were then neutralized to pH ~7 with 8N NaOH, converted to 9-fluorenylmethylchloroformate (FMOC-Cl) derivatives, and analyzed by HPLC using fluorescence detection.

Rates of microbial respiration. Rates of microbial respiration (CO₂ evolution) from collected *Typha* leaves were determined using a LiCor LI-6400 Portable Infrared Gas Analyzer. Collected leaf samples were sectioned into 3-5 ten cm long pieces and placed into a large sterile Petri dish lined with sterile (autoclaved) filter paper. Litter samples and filter paper were wetted with ~30mL of 0.22µm membrane filtered marsh water and incubated in darkness for 2 h at 15 °C. After 2 h of incubation, rates of CO₂ evolution were measured by enclosing plant litter samples into a custom-built Plexiglass chamber connected to the LiCor LI-6400 Infrared Gas Analyzer (see Kuehn et al. 2004). Following respiration measurements, leaf litter samples were stored frozen (-20°C) and later lyophilized and weighed to determine hourly rates of microbial respiration.

Determination of daily and cumulative fungal growth and production rates and microbial respiration rates. Estimates of daily fungal growth rates (μ) and microbial respiration rates were calculated using temperature and relative humidity data obtained from Hobo data loggers placed within the *Typha* stand. These data revealed the diel time periods when litter-associated fungal organisms are likely metabolically active (see Kuehn and Suberkropp 1998a; Kuehn et al. 2004). Fungi colonizing *Typha* leaves were

assumed to be metabolically active during daily time periods where ~100% relative humidity was reached. Consequently, daily fungal growth rates (μ , % / d) and microbial respiration rates were calculated by multiplying the hourly fungal growth rate or microbial respiration rate by the hours per day in which standing *Typha* litter was exposed to ~100% relative humidity conditions. Both fungal growth and respiration rates were also temperature adjusted (assumed $Q_{10} = 2$) to reflect *in situ* field temperatures (see Kuehn et al. 2004; Kuehn et al. 2011). Corresponding rates of daily fungal production were calculated by multiplying the daily growth rate by the litter-associated fungal biomass.

Cumulative fungal production and microbial respiration during standing *Typha* leaf decomposition was calculated by estimating daily rates of fungal production and microbial respiration over the entire annual study period, which followed procedures described earlier by Suberkropp et al. (2010) and Kuehn et al. (2011). Briefly, in order to determine daily fungal production or respiration values for days between monthly sampling dates the following criteria was assumed and calculated: 1) the average hourly fungal growth (μ , % / h) and microbial respiration rate and litter fungal biomass for half of the days between the sampling interval were assumed to be equal to the values obtained on one sampling date, and the hourly fungal growth and microbial respiration rate and litter fungal biomass for the other half of the days in the sampling interval was assumed to be equal to the corresponding values observed on the next sampling date, 2) Daily fungal growth (μ , % / days) and microbial respiration rates were then determined by multiplying the hourly fungal growth and microbial respiration rate by the specific number of hours in that day where the relative humidity was 100%. As above, fungal

growth and respiration rates were temperature adjusted (assumed $Q_{10} = 2$) to account for observed changes in temperatures (see Kuehn et al. 2004; Kuehn et al. 2011), 3) Daily fungal production was subsequently calculated by multiplying the daily growth rate (data not shown) by the litter-associated fungal biomass. This raw data set for the entire sampling period was subsequently used to estimate cumulative fungal production using a Monte Carlo Simulation (see below).

Data analyses. Statistical analyses of the data were conducted using SYSTAT software (version 13), with differences at the $p < 0.05$ level being considered significant. If necessary, data were transformed prior to analysis to reduce heteroscedasticity. Data were analyzed separately using 1-way ANOVA with time as the main factor. Monte Carlo Simulation Analysis using Microsoft Excel PopTools add-ins was used to estimate cumulative fungal production. The raw data set of estimated daily rates of fungal production and microbial respiration over the study period was resampled with replacement to produce 10,000 sets, from which the $\text{mean} \pm 1\text{SD}$ and 95% confidence intervals were calculated. Variance of transformed variables, such as production: biomass ratio and fungal yield were estimated using the delta method (Salkind 2007).

Results

Typha leaf mass loss and nutrient dynamics. During the study period, significant losses in *T. domingensis* leaf mass were observed during the aerial phases of plant senescence and standing decomposition (ANOVA, $F_{11,55} = 6.39$, $p < 0.001$), with ~37% of the leaf carbon mass being lost in 340 days (Figure 9). In August (2008), the initial average area-specific carbon mass of living green *Typha* leaves was $10.79 \pm 0.29 \text{ mgC/cm}^2$ ($\text{mean} \pm 1\text{SE}$, $n=6$). In September, *Typha* leaves began to senesce, with notable yellowing

of leaf tissues proceeding from the leaf tip to the base. By the November sampling date (83 days), *Typha* leaves were fully brown and had an area-specific carbon mass of $9.00 \pm 0.48 \text{ mgC/cm}^2$, corresponding to a mass loss of 16%. Following senescence, gradual decreases in *Typha* leaf mass continued over the study period, with a final leaf-

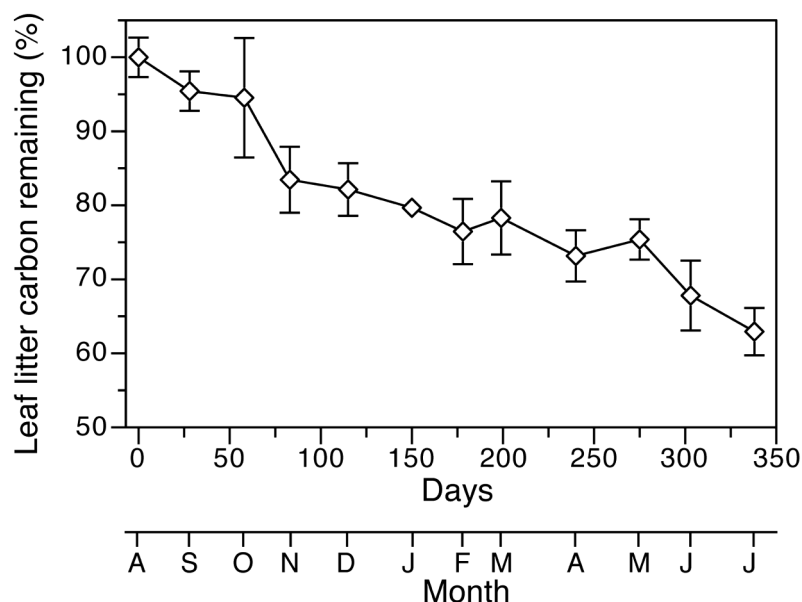


Figure 9. Percent area-specific leaf C remaining of standing *T. domingensis* leaves during the study period. Symbols indicate the mean \pm 1 SE.

area specific carbon mass of $6.79 \pm 0.35 \text{ mgC/cm}^2$ being observed by July 2009. The litter decay rate ($-k/d$), using a non-linear regression model, was -0.0012 ± 0.0002 , and the estimated initial mass N_0 was 98.0 ± 2.4 (values are the mean \pm 1 ASE, regression $r^2 = 0.52$).

Significant decreases in *T. domingensis* leaf nitrogen (N) and phosphorus (P) concentrations (%) were also observed during senescence and standing litter decay (ANOVA, $F_{11,60} = 13.79$ (nitrogen) and 32.97 (phosphorus), $p < 0.001$), which resulted in corresponding increases leaf litter C:N, C:P, and N:P ratios (Figure 10). In August 2008, initial N and P concentrations of living green *Typha* leaves averaged 1.12 ± 0.04 and $0.090 \pm 0.003\%$, respectively. By November, N and P concentrations in *Typha* leaves had

rapidly declined to 0.63 ± 0.03 and $0.032 \pm 0.003\%$, indicating a rapid loss of 45 and 64% of the initial N and P within *Typha* leaves, respectively. After this early decrease, both N and P concentrations increased slightly (to 0.74 ± 0.05 and $0.039 \pm 0.02\%$, respectively), resulting in a slight decrease in litter C:N and C:P ratios (Figure 10A and 10B). However, this trend was followed by further losses in litter nitrogen and phosphorus and a

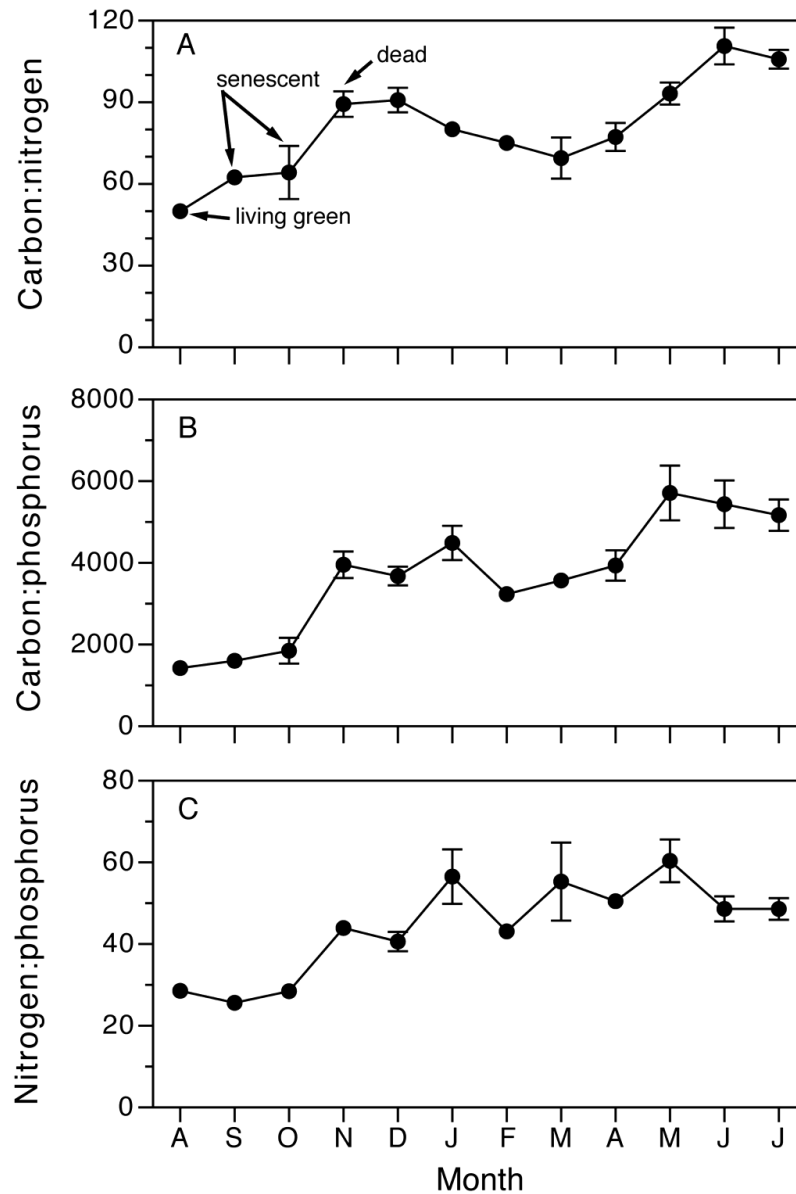


Figure 10. Changes in (A) carbon:nitrogen, (B) carbon:phosphorus, and (C) nitrogen:phosphorus ratios in *T. domingensis* leaves during standing-litter decomposition. Symbols indicate the mean \pm 1SE

corresponding increase in C:N and C:P ratios towards the end of the study period. *Typha* leaf N:P ratios also increase significantly in *Typha* leaves (ANOVA, $F_{(11, 60)} = 15.94$, $p < 0.001$), indicating a disproportionate loss of P versus N during senescence and standing litter decomposition (Figure 10C).

Fungal biomass, production rates and glucosamine. Ergosterol concentrations increased significantly in *Typha* leaves during senescence and standing litter decay (ANOVA, $F_{(11, 59)} = 35.22$, $p < 0.001$). As expected, initial ergosterol concentrations in living green *Typha* leaves (August) were low, averaging 0.037 ± 0.005 mg/g detrital C (Figure 11). Ergosterol concentrations increased markedly with the onset of leaf senescence and decay, with a peak ergosterol concentration of 0.43 ± 0.047 mg/g detrital C being observed in February. Concentrations then declined slightly and remained relatively stable during the remainder of the study period. Corresponding living fungal biomass, estimated from ergosterol concentrations, ranged between 3.16 ± 0.43 and 37.27 ± 4.03 mg C/g detrital C (Figure 11).

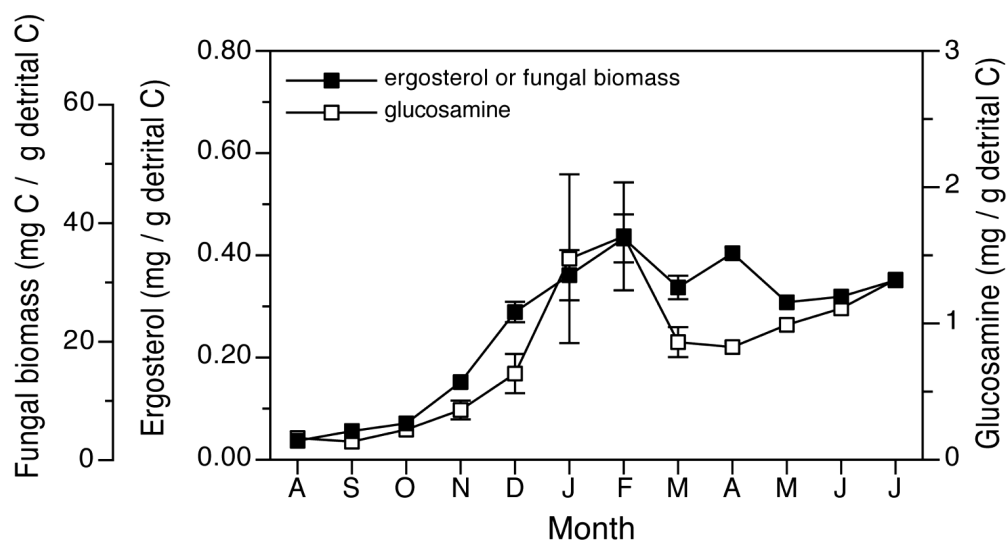


Figure 11. Dynamics of ergosterol and glucosamine concentrations in *T. domingensis* leaves during standing-litter decomposition. Estimated fungal biomass is also indicated. Symbols indicate the mean \pm 1SE.

Significant differences in rates of fungal secondary production (i.e., fungal growth rates $\mu \times$ fungal biomass) associated with *Typha* leaves were observed during the study period (ANOVA, $F_{10,55} = 31.87$, $p < 0.001$). Fungal production rates, based on ^{14}C -acetate incorporation into ergosterol, remained relatively stable during senescence and the early stages of leaf decomposition, ranging between 104 ± 23 and 184 ± 59 $\mu\text{gC/g detrital C/d}$ (Figure 12). Rates then declined in February and remained low until significantly increasing ($p < 0.001$, Tukey) in June and July.

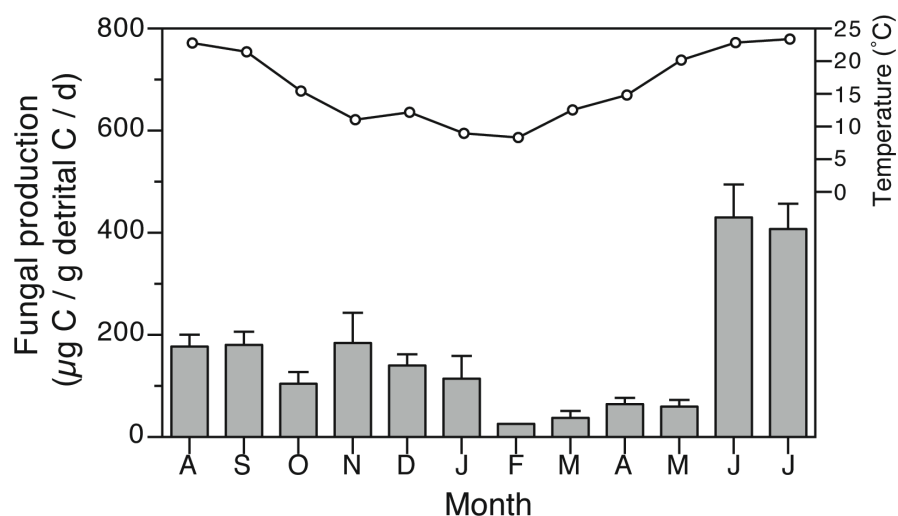


Figure 12. Rates of fungal production associated with *T. domingensis* leaves during standing-litter decomposition. Note: production values for August-October were estimated based on accrual of fungal ergosterol. All other dates were determined based on instantaneous rates of ^{14}C -acetate incorporation. Bars indicate the mean \pm 1 SE. Average daily field temperature ($^{\circ}\text{C}$) on each collection date is also shown.

Changes in chitin (glucosamine) concentrations within *Typha* leaves followed a similar pattern as leaf ergosterol concentrations (Figure 11), with glucosamine increasing significantly within *Typha* leaves during senescence and early standing litter decay. (ANOVA, $F_{(11, 60)} = 5.45$, $p < 0.001$). Average glucosamine and ergosterol concentrations in *Typha* leaf samples were significantly correlated with one another ($r = +0.90$, $p < 0.001$, Pearson).

Like ergosterol, initial concentrations of glucosamine in *Typha* leaves were low but increased markedly during leaf senescence and early decay to a peak concentration of 1.69 ± 0.39 mg C/g detrital C in February. Concentrations declined sharply in March and then gradually increased throughout the remaining study period. Changes in both average ergosterol and glucosamine concentrations within *Typha* leaves were negatively correlated with average area-specific carbon mass ($r = -0.86$ and -0.79 , respectively, $p < 0.001$, Pearson), providing evidence that with increased fungal colonization of *Typha* leaves there is a concomitant loss in *Typha* leaf mass.

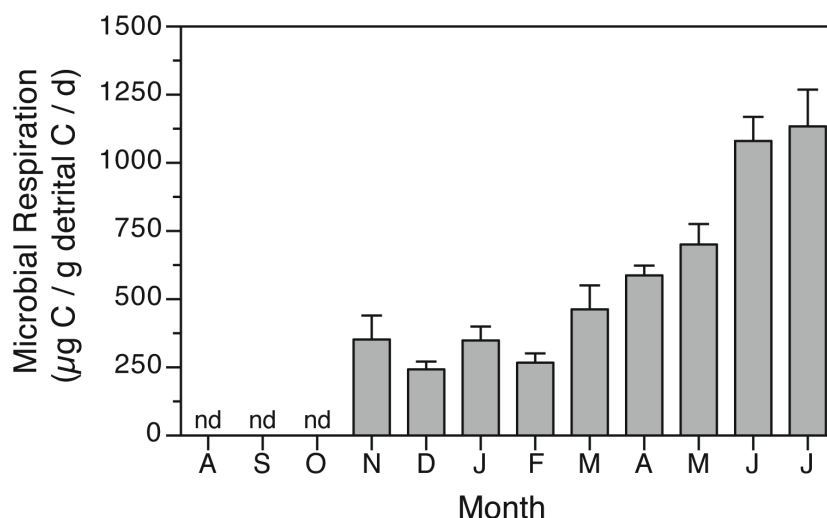


Figure 13. Rates of microbial respiration associated with *T. domingensis* leaves during standing-litter decomposition. nd: no sample measurement were conducted. Bars indicate the mean \pm 1SE.

Microbial community respiration. Rates of microbial respiration (CO_2 evolution) from decaying *Typha* leaves (Figure 13), measured from post-leaf senescence onward, increased significantly during the study period (ANOVA, $F_{(8, 45)} = 19.16$, $p < 0.001$), following a similar pattern, in part, to observed increases in leaf-associated ergosterol (fungal biomass) and glucosamine concentrations (Figure 11). Initial rates of microbial

respiration following leaf senescence were low, averaging $351 \pm 7 \mu\text{gC/g detrital C/d}$. Rates increased steadily throughout litter decomposition, with a peak rate of $1133 \pm 149 \mu\text{gC/g detrital C/d}$ being noted by the end of the study in July 2009. Rates of microbial respiration in *Typha* litter were significantly correlated with rates of fungal production ($r = +0.79$, $p < 0.001$, Pearson), which suggest that most of the observed respiratory activity is likely due to the growth and decay activities of leaf inhabiting fungi.

Fungal nutrient immobilization. Fungal growth and biomass accural within *Typha* leaves during standing litter decomposition (Figure 11) implies that litter-inhabiting fungi may assimilate and immobilize a considerable portion of the detrital nitrogen and phosphorus. Assuming a fungal nutrient stoichiometry of $\text{C}_{106}\text{N}_{16}\text{P}_1$ (Beever and Burns 1980; Findlay et al. 2002; Gulis and Kuehn, unpublished data), estimated detrital nitrogen and phosphorus immobilized by fungi increased during *Typha* leaf decomposition (Figure 14), since increases in observed fungal biomass would required the acquisition and uptake of detrital nutrients to meet fungal stoichiometric demands. The contribution of fungal immobilized N and P reached a maximum of ~40% and ~50% of the total detrital N and P, respectively, during later stages of decay.

Cummulative fungal production and microbial respiration. When integrated over the entire study period, estimated cumulative fungal production using Monte Carlo Simulation analysis totaled $39 \pm 4 \text{ mgC/g initial detrital C}$, indicating that $10.5 \pm 1.2\%$ of the *Typha* leaf carbon was assimilated into fungal biomass (i.e., fungal yield coefficient) during standing litter decay (Table 2). The calculated P/B ratio was 2.3 ± 0.1 , which

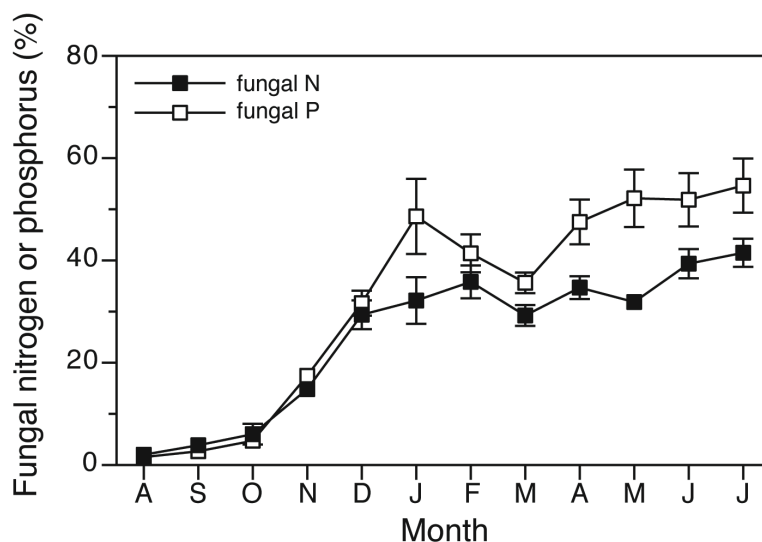


Figure 14. Estimated percentage of total detrital nitrogen and phosphorus immobilized in standing leaf-associated fungal biomass during *T. domingensis* standing-litter decomposition. Symbols indicate the mean \pm 1 SE.

reflects a fungal biomass turnover time of 145 ± 7 days. Corresponding estimates of cumulative microbial respiration (CO_2 evolution) totaled 133 ± 23 mgC/g initial detrital C, providing additional evidence that a considerable amount of *Typha* leaf carbon ($\sim 36\%$) is also mineralized by litter inhabiting microbial decomposers (Table 2). Assuming that microbial respiratory activity was due to fungal organisms, the calculated growth efficiencies of fungi in standing *Typha* litter was 23%.

Discussion

The results of this study reveal significant losses in *T. domingensis* leaf mass and nutrients during standing litter decomposition. During the first 83 days of the study period, *Typha* leaves transitioned from being fully living green to standing-dead (brown), with a corresponding loss in leaf carbon (16%), nitrogen (45%), and phosphorus (64%). Fungal colonization of standing leaves was minor during this initial phase, implying that most of these carbon and nutrient losses were a result of plant translocation or physical

Table 2

Total leaf mass loss, cumulative fungal production, cumulative respiration, mean fungal biomass, P:B ratio, turnover time, and estimated contribution of fungal decomposers to standing- litter decay of T. domingensis leaves. Fungal yield coefficient (%) = cumulative fungal production ÷ total leaf mass loss × 100. The contribution of fungal assimilation to overall carbon loss = (cumulative fungal production + cumulative respiration) ÷ total leaf mass loss × 100. This assumes that all respiratory activity is due to fungal organisms (see Kuehn et al., 2004). Values are the means ± 1SD. Values for cumulative fungal production and respiration rates are the mean±1SD and the 95% confidence interval in parentheses (calculated using Monte Carlo Simulation Analysis).

Parameter	
Total leaf mass loss (mg C / g initial leaf C)	371 ± 39
Cumulative fungal production (mg C / g initial leaf C)	38.9 ± 3.6 (35.2 – 42.8)
Cumulative respiration (mg C / g initial leaf C)	133.4 ± 23.0 (123.8 – 142.9)
Mean fungal biomass (mg C / g initial leaf C)	16.7 ± 0.07 (16.6 – 16.8)
P:B ratio [†]	2.3 ± 0.1
Turnover time (d) [†]	145 ± 7
Fungal yield coefficient (%) [†]	10.5 ± 1.2
Fungal contribution to overall leaf C loss (%) [†]	46.5 ± 5.1

[†]Error estimates were determined using the delta method (Salkind 2007)

leaching during plant senescence. Following senescence, mass loss of *Typha* leaves continued over the remaining study period, with an overall carbon mass loss of 37% being noted after 340 days. During this time, appreciable increases in chitin (glucosamine) and the fungal membrane sterol, ergosterol, were observed in standing *Typha* leaves, indicating the pervasive colonization of standing litter by filamentous fungal decomposers. These findings concur with a growing number of studies conducted in both freshwater and saltmarsh habitats, establishing that initial decomposition of

emergent wetland plants begins in the aerial standing dead phase (Newell and Porter 2000; Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008), well before its collapse to the marsh surface waters or benthic sediments. These initial mass and nutrient loss patterns are complex and involve a combination of both plant and microbial-mediated pathways, which begin with the senescence and death of the emergent plant shoot followed by the colonization and microbial assimilation of carbon and nutrients from plant litter tissues. The results obtained in present investigation and those of former studies also highlight that natural decay patterns of emergent marsh plants is a sequential process that involve two distinct spatial phases separated in time. There is an initial phase resulting from litter decay processes that occur under aerial standing-dead conditions, followed by a second decay phase under submerged or surface sediment conditions after the eventual collapse of standing litter.

Prior studies examining emergent plant decomposition in freshwater marshes have often focused primarily on litter decay processes occurring at, or buried within, the wetland surface sediments (e.g., Poulain 1984; Webster and Benfield 1986; Bayley and Mewhort 2004; Bedford 2005; Rothman and Bouchard 2007; Fennessy et al. 2008). This approach is widespread among many researchers today and is likely grounded in the perception that litter decomposition and associated microbial processes occur exclusively at or within the marsh surface sediments and that sediment-associated bacterial communities are the sole microbial assemblage responsible for its breakdown and mineralization. As a result, the role and contribution of fungal decomposers in marsh biogeochemical processes has not gained general acceptance nor has it been explicitly included in current conceptual or quantitative models describing major wetland carbon

and nutrient flow pathways (Mitsch and Gosselink 2007; Reddy and Delaune 2008). This view is in sharp contrast with studies in freshwater stream systems, where stream dwelling aquatic hyphomycete fungi have been widely documented and recognized as an important microbial assemblage that is central to litter decomposition and detrital food resources for in-habitant consumers (Bärlocher 1992; Gessner and Chauvet 1994; 1997; Suberkropp 1997; Suberkropp 2001; Gulis and Suberkropp 2003; Gulis et al. 2006; Gessner et al. 2007; Chung and Suberkropp 2009; Findlay 2010).

Earlier, Kuehn et al. (2011) examined the senescence and early litter decay patterns of standing *T. angustifolia* leaves in a temperate freshwater marsh in Michigan. Significant transformation and decomposition of *T. angustifolia* litter occurred during the initial standing-decay phase, with a large portion of the plant carbon being channeled into and through litter inhabiting fungal decomposers. During their study, estimated cumulative fungal biomass production during *T. angustifolia* decay totaled 123 mgC/g initial detrital C, indicating that 22% of the observed *Typha* leaf carbon lost (55% or 556 mgC/g initial detrital C) was assimilated into fungal biomass. Furthermore, when integrated over the entire study period, cumulative fungal production estimates were significantly related with cumulative losses in *T. angustifolia* leaf carbon ($r^2 = 0.92$), strongly suggesting that the growth and decay activity of fungal decomposers were likely responsible for most of the observed losses in standing leaf carbon mass.

Similar findings were observed in the present study where an appreciable portion of the *T. domingensis* leaf carbon flowed into and through litter inhabiting fungal communities. During the study period, estimated cumulative fungal production was lower (39 mgC/g initial detrital C) but equated to roughly ~11% of the observed *Typha* leaf

carbon lost (37% or 370 mgC/g initial detrital C). Furthermore, corresponding estimates of cumulative microbial respiration from standing *T. domingensis* leaves totaled 133 mgC/g initial detrital C, which is in accordance with other studies (e.g., Kuehn et al. 2004) indicating that a sizeable fraction of standing leaf litter carbon can also be mineralized by inhabitant microbial assemblages, most likely fungal organisms. When integrated over the entire study period, cumulative increases in fungal production (Figure 15) and microbial respiration (Figure 16) were significantly related with cumulative losses in *Typha* leaf carbon ($r^2 = 0.95$ and 0.83 , respectively), supporting similar observations by Kuehn et al. (2011) and others (Newell and Porter 2000; Gulis et al. 2006; Gessner et al. 2007) that fungi are a quantitatively important driver of emergent litter decomposition during the standing litter decay phase.

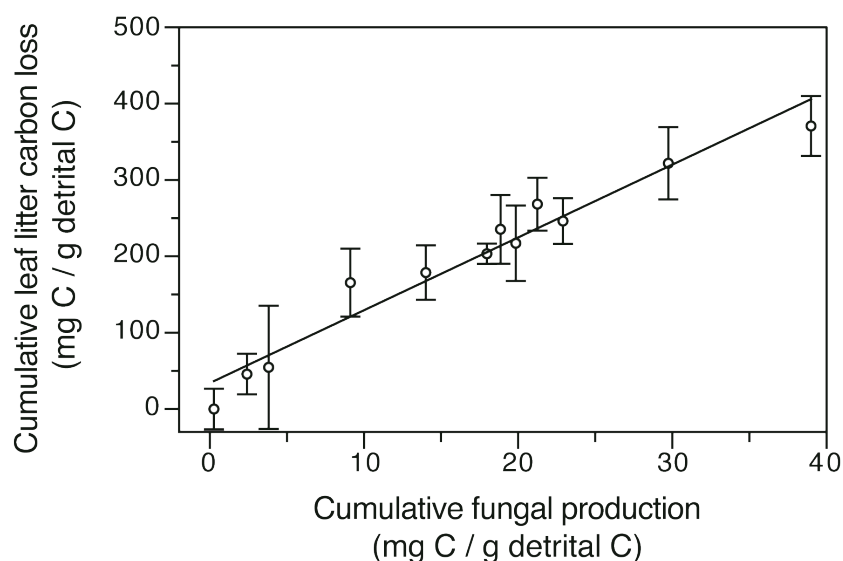


Figure 15. Relationship between cumulative fungal production and cumulative leaf litter carbon loss in standing-dead *T. domingensis* leaves. Linear regression model ($F_{1,10} = 198.38$, $p < 0.001$): Leaf C loss = 9.53 (fungal production) + 34.04 , adjusted $r^2 = 0.95$. Symbols indicate the mean ± 1 SE.

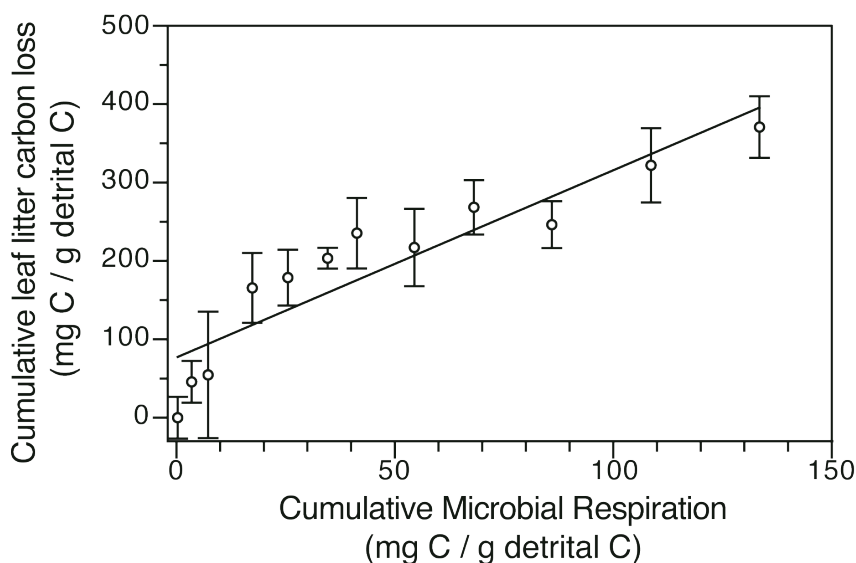


Figure 16. Relationship between cumulative microbial respiration (CO_2 flux) and cumulative leaf litter carbon loss in standing-dead *T. domingensis* leaves. Linear regression model ($F_{1,10} = 53.99$, $p < 0.001$): Leaf C loss = 2.39 (microbial respiration) + 76.79 , adjusted $r^2 = 0.83$. Symbols indicate the mean ± 1 SE.

In addition to assimilating and mineralizing appreciable quantities of carbon within standing litter, fungal decomposers may also be important contributors to the transformation, immobilization, and mineralization of detrital nutrients, such as nitrogen and phosphorus. Assuming a nutrient stoichiometry of $\text{C}_{106}\text{N}_{16}\text{P}_1$ in fungal biomass (Beever and Burns 1980; Findlay et al. 2002; Gulis and Kuehn unpublished data), fungal communities inhabiting *T. domingensis* immobilized a sizeable fraction of the detrital nitrogen and phosphorus during standing litter decomposition, reaching a maximum of ~40% and ~50% of the total detrital N and P during later stages of decomposition. Similar findings have also been noted in other studies (Findlay et al. 2002, van Ryckegem et al. 2006; Kuehn et al. 2011), suggesting that standing litter fungal communities may also play an important role in marsh nutrient cycling pathways.

A notable and unexpected finding in the present study is the observation of much lower rates of mass loss and fungal biomass production associated with standing *T. domingensis* litter in this subtropical freshwater marsh site versus similar comparative estimates from standing *Typha* litter in temperate marsh systems. I had predicted that the contribution of fungal decomposers would be higher in subtropical versus temperate freshwater marshes (e.g., Kuehn et al. 2011) due to higher temperatures, relative humidity patterns, and a much longer growing season. In the present study, *T. domingensis* at the Weeks Bay subtropical marsh site lost 37% of the leaf carbon mass over the annual study period (340 days), with corresponding rates of fungal production ranging between 25 and 430 $\mu\text{gC/g detrital C/d}$ and living fungal biomass reaching a maximum of 37 mg C/g detrital C. In comparison, Kuehn et al. (2011) observed over 55% mass loss in 350 days of standing-dead *T. angustifolia* leaves in a temperate freshwater marsh. During their study, rates of fungal production associated with standing-dead litter were much higher, ranging between 87 and 3126 $\mu\text{gC/g detrital C/d}$. In addition, *T. angustifolia* leaves accumulated significantly higher concentrations of fungal biomass.

The apparent differences in the performance and overall contribution of fungi to *Typha* decomposition between these subtropical and temperate freshwater marshes may be reflected in the intrinsic nutrient quality of *Typha* plant litter substrate, which can impact the ability of fungal decomposers to meet their stoichiometric demands for growth and reproduction (Sinsabaugh and Follstad-Shah 2012; Sinsabaugh et al. 2013). In the present study, significant decreases in litter-associated nitrogen and phosphorus were observed in standing *T. domingensis* leaves during the study period, resulting in average

C:N and C:P ratios of 88 and 4352 during the post-senescent stages of decomposition, respectively. In contrast, Kuehn et al. (2011) observed an average C:N and C:P ratio of 67 and 2583, respectively, during standing litter decay of *T. angustifolia*, which suggests a greater availability of detrital nitrogen and phosphorus sources for litter inhabiting fungal decomposers. Prior studies have documented that detrital nutrient concentrations as well as increased exogenous nutrient supplies can significantly influence the growth and decay activities of fungal decomposers (Gulis and Suberkropp, 2003).

Emergent vascular plants are an important structural component in freshwater marshes and decomposition of their litter is widely regarded as a key process that drives marsh ecosystem carbon and nutrient cycling. Results obtained in this study provide additional data to our quantitative understanding of the role and contribution of fungi in marsh carbon and nutrient cycling pathways. In freshwater marshes where plant litter decomposition has been studied under natural conditions, fungal decomposers are continuing to emerge as an important microbial assemblage, which should be integrated into ecosystem models that depict important marsh carbon and nutrient flow pathways.

CHAPTER IV
ECOSYSTEM SCALE RESPIRATION AND ANNUAL FUNGAL PRODUCTION
ASSOCIATED WITH STANDING-DEAD LITTER IN A CLADIUM/JUNCUS
FRESHWATER MARSH

Introduction

In freshwater marshes, emergent vascular plants, such as *Juncus* and *Typha*, often account for a large fraction of the plant biomass produced on an annual basis (Kvêt and Westlake 1998; Wetzel 2006; Mitsch and Gosselink 2007). These plants exhibit prolific rates of growth and nutrient (nitrogen and phosphorus) sequestration, with estimates of aboveground biomass production alone frequently exceeding 1000 g/m²/y. As a result, emergent marsh plants embody an important ecosystem reservoir of carbon and nutrients, which are usually depicted as the principal carbon and nutrient pools in most wetland elemental budgets (Mitsch and Gosselink 2007; Reddy and Delaune 2008).

Most of this emergent plant production enters the detrital pool following plant senescence and death, where microbial decomposers and detritus-feeding consumers play an important role in its decomposition and mineralization (Moore et al. 2004; Hagen et al. 2012). During decomposition, microorganisms colonize, enzymatically degrade, and assimilate plant litter carbon and nutrients (e.g., nitrogen and phosphorus) to meet their metabolic and stoichiometric demands for growth and reproduction (Sinsabaugh and Follstad-Shah 2012; Sinsabaugh et al. 2013). In turn, this microbial decay activity results in transformation, breakdown, and conversion of plant litter carbon and nutrients into microbial biomass, CO₂ via the microbial respiratory mineralization of organic matter, or its export as dissolved (DOM) or fine particulate organic matter (FPOM).

In many freshwater emergent macrophytes, the collapse of plant shoots to the sediments or overlying surface waters does not typically occur following senescence and death, resulting in the accumulation of standing-dead litter (Asaeda et al. 2002; Christensen et al. 2009). Diverse assemblages of fungi are known to pervasively colonize and reproduce on and within standing litter in both subtropical and temperate climates (e.g., Gessner and van Ryckegem 2003), where they are early participants in the litter decomposition process (Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008). Appreciable accumulation of fungal biomass has been observed in standing-dead litter (Newell et al. 1995; Bärlocher and Biddiscombe 1996; Kuehn and Suberkropp 1998b; Kuehn et al. 1999; Gessner 2001; Findlay et al. 2002; Newell 2003; Verma et al. 2003; Welsch and Yavitt 2003; Kuehn et al. 2011), indicating that litter inhabiting fungal decomposers are effective in enzymatically acquiring and assimilating plant litter C and nutrients to support their pervasive mycelial growth and the production of reproductive structures (e.g., ascoma, see Newell 2001b).

Recently, Kuehn et al. (2011) quantified the biomass and secondary production rates of fungi within standing-dead *Typha angustifolia* litter in a north temperate freshwater marsh. Significant mass loss of *T. angustifolia* litter occurred in the standing-dead position (~55%), with a large portion of the plant litter carbon (22%) and nutrients (>50% N and P) being channeled into fungal biomass during decomposition. Additional studies have also documented that microbial communities, predominantly fungi, are well adapted to the harsh conditions (i.e., cyclic water stress availability) in standing litter environment (Kuehn et al. 1998) and can mineralize an appreciable portion of the standing litter C before its collapse to the overlying surface waters of marsh sediments

(Kuehn and Suberkropp 1998a; Welsch and Yavitt 2003; Newell 2003; Kuehn et al. 2004; Zhang et al. 2014).

Currently, most studies examining fungal dynamics in freshwater emergent plant litter have focused on quantifying biomass and production rates during specific litter decay periods. Plant litter of known age and type was placed in litter bags or tagged and sampled through time (e.g., Newell et al. 1995; Bärlocher and Biddiscombe 1996; Kuehn and Suberkropp 1998; Kuehn et al. 1999; Kuehn et al. 2000; Komínková et al. 2000; Gessner 2001; Menéndez 2008; Kuehn et al. 2011). This approach has been useful in constructing partial decay budgets that assess the quantitative contribution of fungal decomposers to standing or submerged litter mass loss (Kuehn et al. 2000; Komínková et al. 2000; Gessner 2001; Kuehn et al. 2011). However, to quantify the contribution of fungi or other microbial assemblages (bacteria) to ecosystem-scale carbon and nutrient cycling, it is necessary to have areal estimates of microbial biomass and production associated with naturally-occurring plant litter, which would include plant litter in various decomposition stages (see Suberkropp 1997).

To date, only three estimates of this kind are available for litter decomposing fungi in freshwater marsh ecosystems (Buesing and Gessner 2006; Kuehn 2008; Ohsowski 2008), and only one exists for saltmarsh ecosystems (Newell 2001a). Earlier, Ohsowski (2008) estimated areal annual bacterial and fungal production and community respiration rates associated with both standing and benthic *T. angustifolia* litter in a north temperate freshwater marsh. Overall, annual microbial production was dominated by litter associated fungal decomposers, with ~9.6% of the annual *T. angustifolia* plant production (1110 g/m²/y) flowing to (biomass) and through (CO₂ respiratory flux) the

microbial communities inhabiting standing litter. These findings suggest that fungal decay processes may be an important pathway of ecosystem carbon and nutrient flow in freshwater marshes.

The present study was conducted to determine annual areal rates of microbial respiration (CO₂) and fungal production associated with standing-dead litter in a subtropical freshwater *Cladium-Juncus* marsh. Living and dead plant litter standing crops, fungal biomass and production, and microbial respiration rates were determined at monthly intervals over an annual period and used to construct a budget to estimate annual fungal contributions to ecosystem-level (g/m²) carbon flow in the standing-dead litter compartment. In addition, the biomass dynamics of fungal decomposers relative to changes in litter nitrogen and phosphorus standing stocks were also examined in order to assess the potential importance of fungal decomposers to ecosystem-level nutrient cycling. Overall, I predicted that the fungal contribution to ecosystem carbon and nutrient cycling in standing litter would be greater in a subtropical freshwater marsh versus a temperate marsh (e.g., Ohsowski 2008) due to the presence of more conducive environmental conditions for fungal growth and respiration (e.g., higher temperatures, relative humidity, longer growing season).

Study Site

This study was conducted in 10.18 ha freshwater emergent marsh located in the Weeks Bay National Estuarine Research Reserve (NERR) near Magnolia Springs, Alabama, USA (N30°25.39, W87°49.26). The marsh site, referred to as Southern marsh, is dominated by a mix of *C. jamaicense* and *J. roemerianus* and is situated within an extensive complex of other marshes also dominated by *C. jamaicense* and *J.*

roemerianus. *Typha domingensis* is also present within the marsh complex but is much less abundant and restricted to certain locations. Collectively, the marsh complex is located near the mouth of the Fish River as it enters the northern portion of Weeks Bay.

Materials and Methods

Field sampling procedures. Naturally-occurring aboveground plant mass was collected monthly over an annual period. On each sampling date, destructive samples of both living and standing-dead plant mass were collected in six randomly selected sub-plots (0.0625 m^2) along a 100 meter transect extending through the mixed emergent *Cladium/Juncus* marsh stand. Collected plant material was placed into clean plastic garbage sacks and immediately returned to the laboratory. In the laboratory, aboveground plant material was separated into living and dead fractions, and standing-dead litter fractions were randomly subsampled for fungal biomass and production and microbial respiration assays (see below). The remaining living and dead plant fractions were sectioned into smaller pieces, placed into clean brown paper bags, dried (105°C), and weighed to determine the amount of living and standing-dead plant mass per m^2 of wetland surface area. Subsamples of dried plant material were later ground to a powder using a Wiley mill (40-mesh, $\sim 425 \text{ }\mu\text{m}$ particle size) and analyzed for carbon, nitrogen, and phosphorus concentrations. Carbon and nitrogen concentrations in ground plant samples were determined using a Costech 4010 elemental combustion analyzer. Corresponding phosphorus concentrations in plant samples were determined using a SEAL AA3 Flow Injection Nutrient Analyzer (molybdate-ascorbic acid method) following combustion (500°C) and hot HCl extraction of ground plant subsamples.

Air temperatures and relative humidity were continuously recorded at 15 minute intervals throughout the entire study period using two Onset Hobo H8 Pro series data loggers, which were placed at mid-canopy height directly within marsh stand (Figure 17). Precipitation data was also recorded via a permanent meteorological data station (Figure 17), which was located near the marsh stand and was operated and maintained by the Weeks Bay Reserve. Surface water samples of the Fish river adjacent to the marsh were

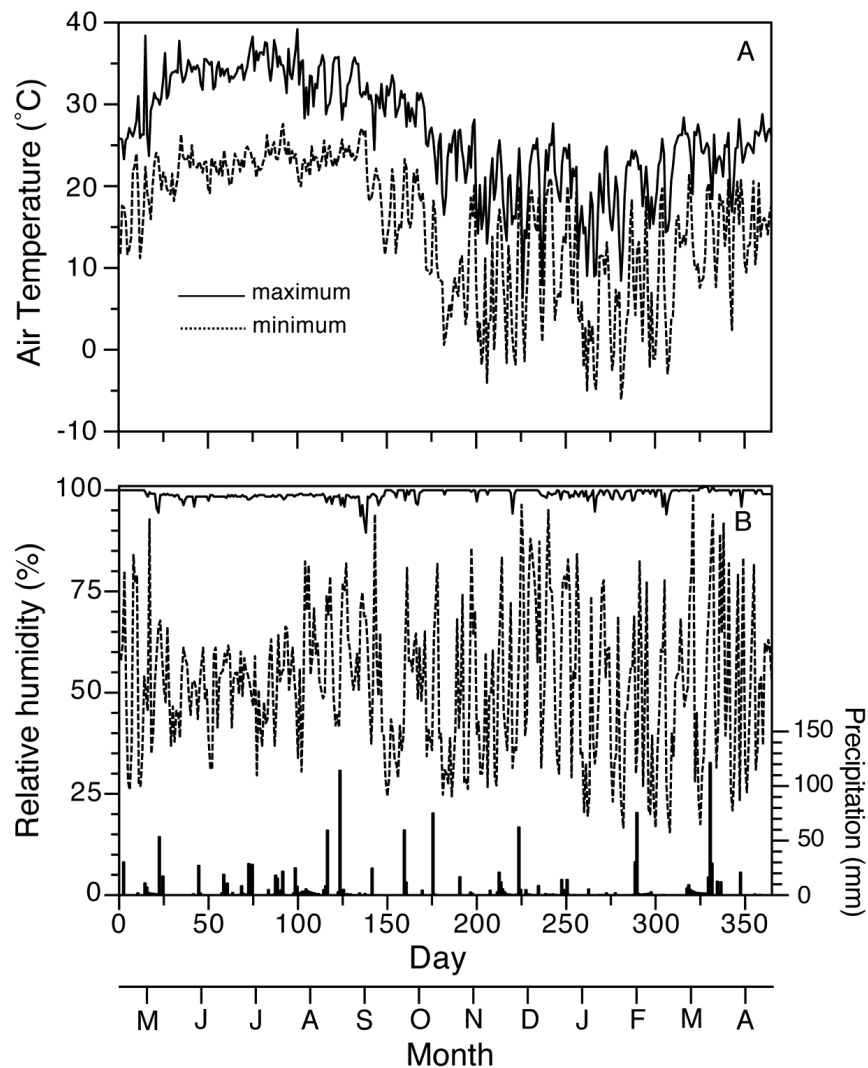


Figure 17. Changes in maximum (solid line) and minimum (dashed line) (A) air temperature, and (B) relative humidity and daily total precipitation (dark vertical bars) in the *C. jamaicense* and *J. roemerianus* marsh stand during the annual study period.

Table 3

Selected characteristics of Fish River water and the Cladium/Juncus marsh stand where annual fungal production and microbial respiration studies were conducted. Values are the means \pm 1SD, with ranges in parentheses.

Parameter	
<i>Cladium/Juncus</i> marsh surface area (ha)	10.18 ha
Mean annual living plant biomass (g C / m ²)	389 \pm 112 (253-542)
Mean annual standing-dead plant mass (g C / m ²)	643 \pm 104 (496-819)
Shoot density (# / m ²)	480 \pm 145 (235-693)
pH	7.5 \pm 0.8 (5.5-9.6)
Conductivity (mS / cm)	12.6 \pm 8.0 (263-373)
Salinity (ppt)	7.4 \pm 5.0 (0-17.5)
SRP (μ g / L)	4 \pm 2 (1-5)
Total P (μ g / L)	18 \pm 9 (2-35)
NH ₄ ⁺ -N (μ g / L)	70 \pm 28 (20-158)
NO ₃ ⁻ -N (μ g /L)	498 \pm 182 (97-1031)

collected on each sampling date, placed on ice, and returned to the laboratory for analysis of concentrations of dissolved nutrients (TP, NO₂-NO₃, NH₄). In addition, surface water samples were also monitored bi-monthly by Weeks Bay Reserve scientists for dissolved nutrient concentrations of (SRP, NO₂-NO₃, NH₄) and continually monitored at 15 minute intervals for surface water temperatures, specific conductivity, salinity, and pH using

YSI6600EDS multiparameter sonde dataloggers. All meteorological and water quality data collected by the Weeks Bay Reserve is available online (<http://cdmo.baruch.sc.edu/>) at the National Estuarine Research Reserve System's Centralized Data Management Office. Selected characteristics of Fish River surface waters that flow into the marsh complex are summarized in Table 3.

Fungal biomass and rates of fungal production. The biomass and growth rates of fungi associated with naturally-occurring standing-dead plant litter were estimated from litter ergosterol concentrations and rates of [$1\text{-}^{14}\text{C}$]-acetate incorporation into ergosterol, respectively (Gessner 2005; Suberkropp and Gessner 2005). Subsamples (3-5 ~2-cm pieces) of standing-litter fractions from each plot were incubated in sterile 20 ml glass scintillation vials containing 4 ml of filtered (0.22 μm membrane filter) Fish River marsh water and 5 mM Na[$1\text{-}^{14}\text{C}$]-acetate (specific activity = 48 Mbq/mmol, MP Biomedicals Inc.) for 4-5 h at 15°C. One additional vial containing litter sections and formalin (2% v/v final concentration) was also included to correct for any non-biological incorporation. Incorporation of [$1\text{-}^{14}\text{C}$]-acetate label in samples was stopped by placing vials on ice and immediately filtering (1.2- μm glass fiber filter) the contents. Filters and litter pieces were washed twice with 4 ml of filtered marsh water, placed back in scintillation vials, and stored frozen at -20 °C until analyzed. Later, frozen samples were lyophilized, weighed, and ergosterol extracted in alcoholic KOH (0.8% KOH in HPLC grade methanol, total extraction volume 10 ml) for 30 minute at 80°C in tightly capped thick-walled digestion tubes. The resultant crude extract was partially cleaned by solid-phase extraction and quantified using a Shimadzu High Pressure Liquid Chromatograph system (see Kuehn et al., 2011). Injected ergosterol fractions eluting from the HPLC were collected in clean 20

ml glass scintillation vials using an Advantec (SF-3120) fraction collector system, mixed with 10 ml of Ecolume scintillation fluid, and radioactivity determined using a Beckman LS6500 Scintillation Counter. Sample radioactivity was corrected for quenching and background radioactivity that was observed in corresponding killed controls. Litter associated ergosterol concentrations and radioactivity were determined as the average of two HPLC injections per sample extract.

Estimates of living fungal biomass were inferred from ergosterol concentrations using a conversion factor of 5 μg ergosterol/mg fungal dry mass, and 43% carbon in fungal dry mass. Estimates of hourly fungal growth rates (μ , % / h), based on rates of [^{14}C]-acetate incorporation into ergosterol, were calculated using a conversion factor of 12.6 μg fungal biomass synthesized / nmole acetate incorporated. Corresponding hourly rates of fungal production were determined by multiplying the fungal growth rate by the fungal biomass concentration.

Rates of microbial respiration. Rates of microbial respiration (CO_2 evolution) from standing litter subsamples were determined using a LiCor LI-6400 Infrared Gas Analyzer. Standing-litter subsamples (3-5 ~10-cm pieces) from each plot were placed into a large sterile Petri dish lined with sterile (autoclaved) filter paper. Litter samples and filter paper were wetted with ~30mL of 0.22 μm membrane filtered marsh water and incubated in darkness for 2 h at 15 °C. After 2 h of incubation, rates of CO_2 evolution were measured by enclosing plant litter samples into a custom-built Plexiglass chamber connected to the LiCor LI-6400 Infrared Gas Analyzer. Following respiration measurements, leaf litter samples were placed back into Petri dishes, stored frozen (-20 °C), and later lyophilized and weighed to determine hourly rates of microbial respiration.

Determination of daily fungal growth, production, and microbial respiration rates in standing litter. Estimates of daily fungal growth rates (μ) and microbial respiration rates were calculated using temperature and relative humidity data that was obtained from Hobo data loggers placed within the *Cladium/Juncus* marsh stand, which revealed the diel time periods when litter-associated fungal decomposers were likely metabolically active and growing (see Kuehn and Suberkropp 1998a; Kuehn et al. 2004). Fungi colonizing aerial standing litter were considered metabolically active during daily time periods where $\sim 100\%$ relative humidity was reached. Consequently, estimated daily fungal growth rates (μ , % / d) and microbial respiration rates were calculated by multiplying the hourly fungal growth rate or microbial respiration rate, determine in the laboratory, by the timeframe (hours per day) where standing litter was exposed to $\sim 100\%$ relative humidity conditions. In addition, fungal growth and respiration rates determined under laboratory temperature conditions ($15\text{ }^{\circ}\text{C}$) were also adjusted (assuming a $Q_{10} = 2$) to reflect in situ field temperatures (see Kuehn et al. 2004; Kuehn et al. 2011). Corresponding daily rates of fungal production were calculated by multiplying this humidity adjusted daily growth rate (μ , % / d) by the litter fungal biomass concentration.

Calculation of annual areal fungal production and microbial respiration.

Cumulative estimates of areal annual fungal production and microbial respiration were calculated by interpolating between sampling dates and summing up daily production or respiration estimates over the year, which followed similar protocols published earlier (see Suberkropp 1997; Methvin and Suberkropp 2003; Carter and Suberkropp 2004; Suberkropp et al. 2010). Briefly, in order to determine daily areal fungal production or respiration values for the interval between monthly sampling dates the following criteria

was assumed and calculated: (1) The average estimates for fungal growth rates (μ , % / hour), fungal biomass (mgC/g detrital C), microbial respiration rates (mgC-CO₂/g detrital C/h), and litter standing stocks (gC/m²) for half of the days between sampling dates were assumed to be equal to the values obtained on one sampling date, and the fungal growth rates, fungal biomass, microbial respiration, and litter standing stocks for the other half of days in the sampling interval were assumed to be equal to the corresponding values observed on the next sampling date. (2) As above, daily fungal growth (μ , % / days) and microbial respiration rates (mgC-CO₂/g detrital C/d) were determined by multiplying the hourly fungal growth and microbial respiration rate by the specific number of hours in that particular day where the relative humidity was 100%, as provided by Hobo data loggers. (3) Daily fungal production was subsequently calculated by multiplying this daily adjusted fungal growth rate (μ , % / days) by the litter-associated fungal biomass concentration. As above, fungal growth and respiration rates were also temperature adjusted (assumed $Q_{10} = 2$) to account for observed changes in in situ temperatures. (4) Microbial respiration and fungal production on an areal basis for each day were then calculated by multiplying the daily microbial respiration and fungal production rate (gC/g detrital C/d) by the corresponding litter standing stocks (gC/m²). This raw data set was then used to estimate annual areal fungal production and respiration using a Monte Carlo Simulation (see below).

Data analyses. Statistical analyses of the data was conducted using SYSTAT (version 13), with differences at the $p < 0.05$ level being considered significant. If necessary, data were transformed prior to analysis to reduce heteroscedasticity. Data were analyzed separately using a one-way ANOVA with time as the main factor or by using a

Student's t-test when only two means were compared. Monte Carlo Simulation Analysis was conducted using Microsoft Excel with PopTools add-ins. The raw annual data set of estimated daily fungal production or microbial respiration per m² marsh surface was resampled with replacement to produce 10,000 sets, from which the mean \pm 1SD and 95% confidence intervals were calculated. Variance of transformed variables, such as the estimated annual production: biomass ratio, turnover times, and fungal growth efficiencies, were estimated using the delta method (Salkind, 2007).

Results

Living plant mass and standing-dead litter standing stocks. Aboveground living plant biomass collected during the study period averaged 389 ± 112 gC/m² (\pm 1SD, n=12), which includes both *C. jamaicense* and *J. roemerianus* shoots (Table 3). Total living plant biomass fluctuated throughout the study period with higher areal biomass estimates being observed during the peak growing season in summer and lower estimates being noted in the fall and winter (Figure 18); however, the observed differences were not

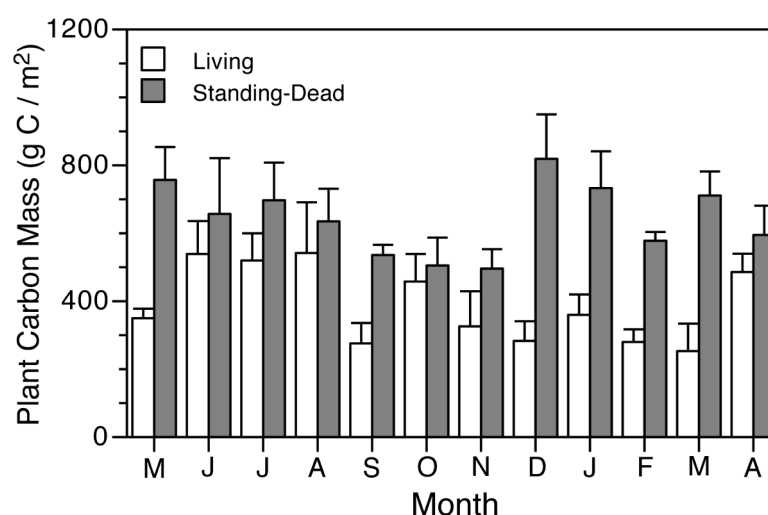


Figure 18. Annual dynamics of living and standing-dead plant carbon mass in the *C. jamaicense* and *J. roemerianus* marsh stand during annual study period. Bars indicate the mean \pm 1SE.

significant (ANOVA, $F_{11,60} = 1.94$, $p=0.052$). Over the annual study period, mean estimates of areal standing-dead plant litter mass were significantly higher (Student's t -test, $t_{11} = -5.49$, $p<0.001$) than living plant biomass, averaging $643 \pm 104 \text{ gC/m}^2$. In contrast to living plant biomass, higher amounts of standing-dead litter mass were observed in the fall and winter compared to the summer (Figure 18), but these differences were not significant (ANOVA, $F_{11,60} = 1.16$, $p=0.332$).

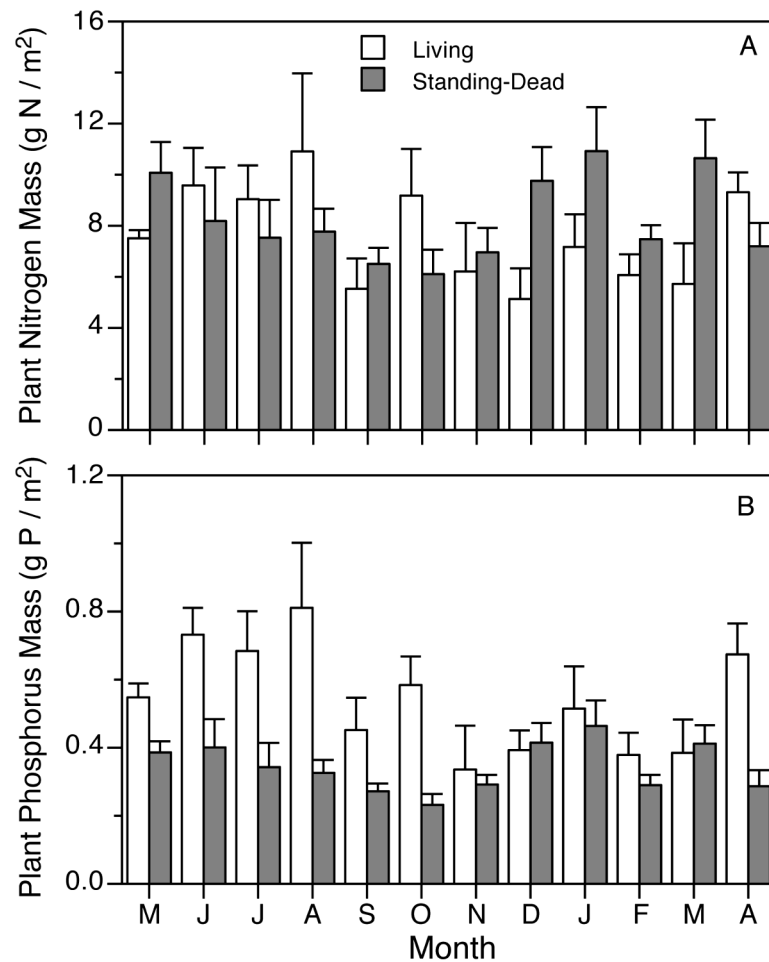


Figure 19. Annual dynamics of (A) nitrogen and (B) phosphorus in living and standing-dead plant matter in the *C. jamaicense* and *J. roemerianus* marsh stand during annual study period. Bars indicate the mean \pm 1 SE.

Standing stocks of plant-associated nutrients indicate that standing-dead litter is a quantitatively important ecosystem pool nitrogen (N) and phosphorus (P) (Figure 19). Levels of N in both living and standing-dead plant matter were similar throughout the annual study period (Student's t-test, $t_{11} = -0.77$, $p=0.46$), with N mass averaging 7.6 ± 1.9 and 8.3 ± 1.7 gN/m² (± 1 SD, $n=12$), respectively. Nitrogen standing stocks in both living and standing-dead plant matter remained relatively stable throughout the study (Figure 19), with no significant differences observed among sampling dates (ANOVA, $F_{11,60} = 1.55$ and 1.72 , $p=0.14$ and 0.09 , respectively). In contrast to plant associated N, P standing stocks in living and standing-dead plant matter were significantly different (Student's t-test, $t_{11} = 3.91$, $p<0.01$), with annual P mass in living biomass averaging 0.54 ± 0.16 gP/m² compared to 0.34 ± 0.07 gP/m² in standing-dead litter. Phosphorus in living plant biomass fluctuated significantly over the study period (ANOVA, $F_{11,60} = 2.26$, $p<0.05$), with higher levels of P being observed during the summer growing season, which coincided with peak levels of living plant biomass (Figure 18). In comparison, P mass in standing-dead litter remained relatively stable throughout the study, with no significant differences being observed among sampling dates (ANOVA, $F_{11,60} = 1.92$, $p=0.06$).

Fungal biomass, production, and nutrient immobilization. Living fungal biomass per gram of plant litter carbon, as determined from litter ergosterol concentrations, remained fairly constant over the study period averaging 30 ± 5 mgC/g detrital C (± 1 SD) or $\sim 3\%$ of the total detrital weight (Figure 20). No significant fluctuations in fungal biomass concentrations were noted during the study period (ANOVA, $F_{11,60} = 0.89$, $p=0.55$). Corresponding fungal biomass concentrations per square meter of marsh surface

followed a similar pattern (Figure 20), with no significant fluctuation being observed (ANOVA, $F_{11,60} = 0.72$, $p=0.72$). Mean fungal biomass over the entire annual study period was 18 ± 3 gC/m² (± 1 SD, range 15-25).

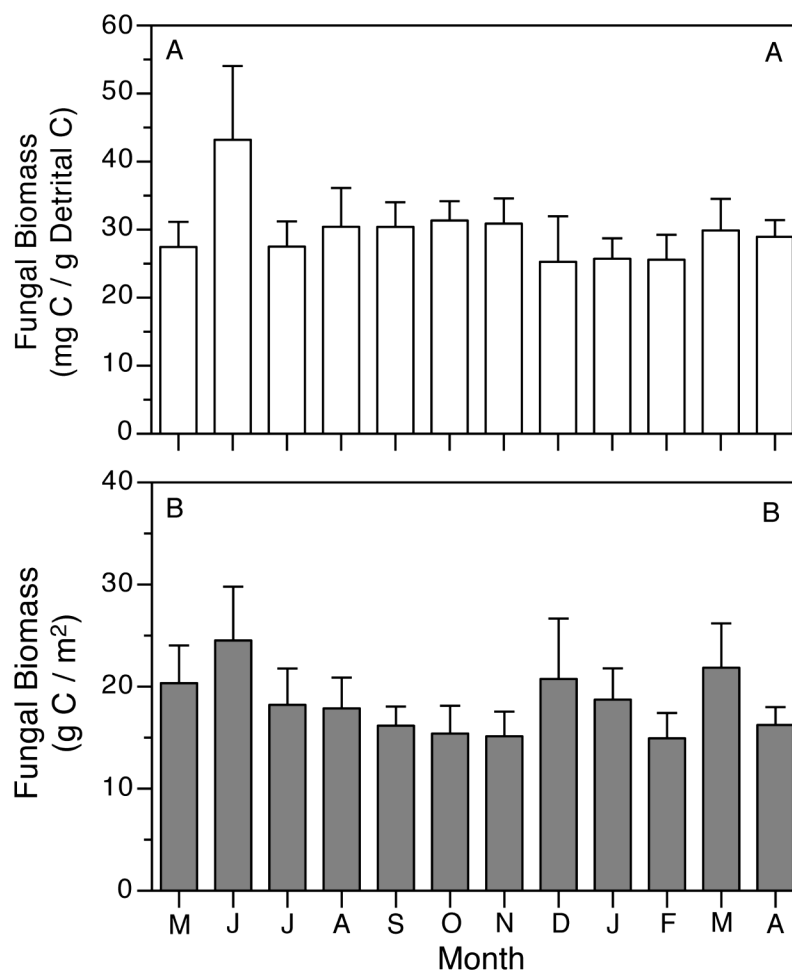


Figure 20. Fungal biomass (ergosterol) (A) per gram of standing detrital carbon and (B) per m² of marsh surface area in the *C. jamaicense* and *J. roemerianus* marsh stand during the annual study period. Bars indicate the mean ± 1 SE.

Daily rates of fungal secondary production per gram of plant litter carbon varied significantly during the study period (ANOVA, $F_{11,58} = 9.08$, $p < 0.001$), with higher rates being observed in the summer versus the winter (Figure 21). Rates ranged between 0.02 ± 0.005 to 1.91 ± 0.43 mg C/g detrital C/d (± 1 SE) and were similar to changes in

temperatures observed on individual sampling dates. Corresponding rates of fungal production per square meter of marsh followed a similar pattern, with significant fluctuations in rates being noted during the study period (ANOVA, $F_{11,58} = 7.21$, $p < 0.001$). Rates ranged between 12 ± 3 to 1106 ± 225 mg C/m²/d, (± 1 SE), with higher areal rates being observed during the summer. When integrated over the entire study period, estimated annual fungal production totaled 90 ± 4 gC m² (Table 4), which is equivalent to a fungal assimilation of $\sim 14\%$ of mean annual standing litter mass (643 ± 104 gC/m²).

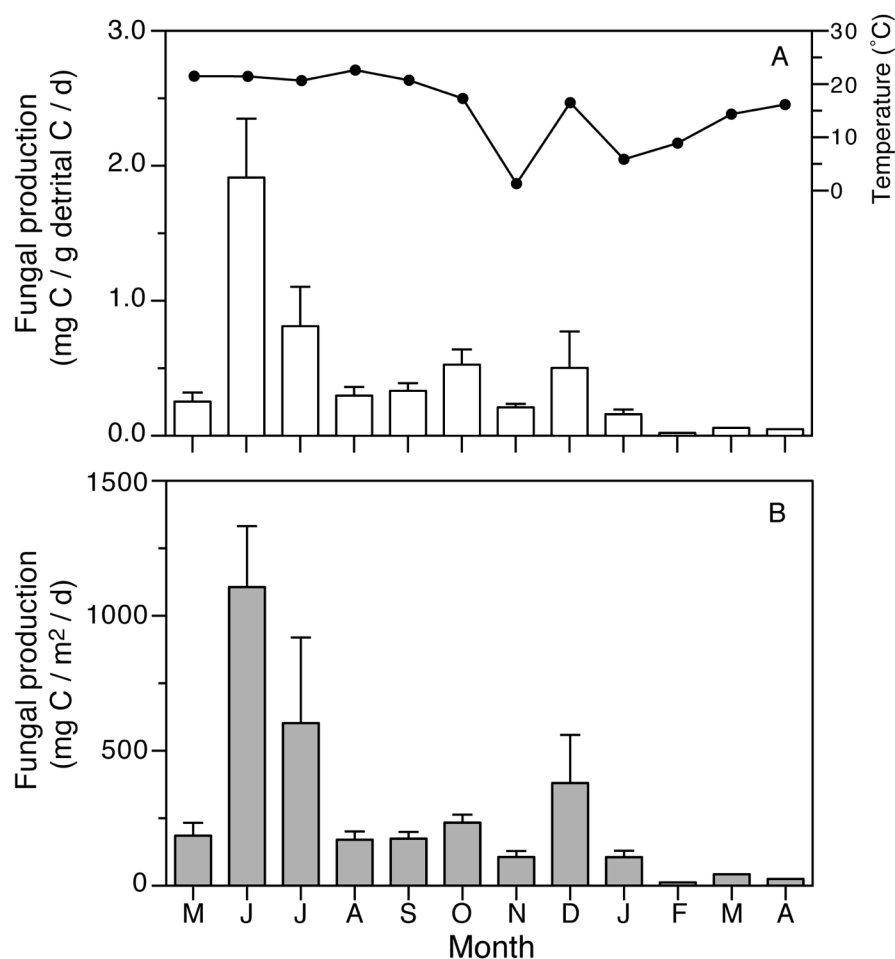


Figure 21. Fungal production (¹⁴C-acetate incorporation to ergosterol) (A) per gram of standing detrital carbon and (B) per m² of marsh surface area in the *C. jamaicense* and *J. roemerianus* marsh stand during annual study period. Bars indicate the mean ± 1 SE. Average daily field temperature (°C) on each collection date is also shown.

Table 4

Annual fungal production and respiration associated with naturally occurring standing-dead plant litter in the C. jamaicense and J. roemerianus the marsh stand. The mean annual fungal biomass, P:B ratio, turnover time, and estimated fungal growth efficiency is also given. Fungal growth efficiency (%) = annual fungal production ÷ (annual fungal production + annual respiration) × 100, which assumes that all respiratory activity is due to fungal decomposers. Values are the means±1SD. Values for annual fungal production and respiration represent the means±1SD and the 95% confidence interval in parentheses (calculated using Monte Carlo Simulation Analysis).

Parameters	
Mean annual fungal biomass (g C/m ²)	18±3
Annual fungal production (g C/m ² /y)	90±4 (82-99)
Annual respiration (g C/m ² /y)	124±5 (114-135)
P:B ratio [†]	4.9±0.8
Turnover time (d) [†]	74±13
Annual growth efficiency (%) [†]	42±3

[†]Error estimates determined using the delta method (Salkind 2007).

The estimated annual production to biomass ratio (P/B) was 4.9±0.8, indicating a biomass turnover time of 74±13 days in the standing litter compartment (Table 4).

The production and subsequent biomass accumulation of fungal decomposers in standing-dead litter also indicates that litter-inhabiting fungi acquire and immobilize a considerable portion of the total detrital N and P into fungal biomass. Assuming a fungal nutrient stoichiometry of C₁₀₆N₁₆P₁ (Beever and Burns 1980; Findlay et al. 2002; Gulis and Kuehn unpublished data), estimated detrital nitrogen and phosphorus pools (Figure

19) immobilized into fungi biomass (Figure 20) averaged 35 ± 6 and $51\pm7\%$ of the total detrital N and P in standing litter, respectively (Figure 22).

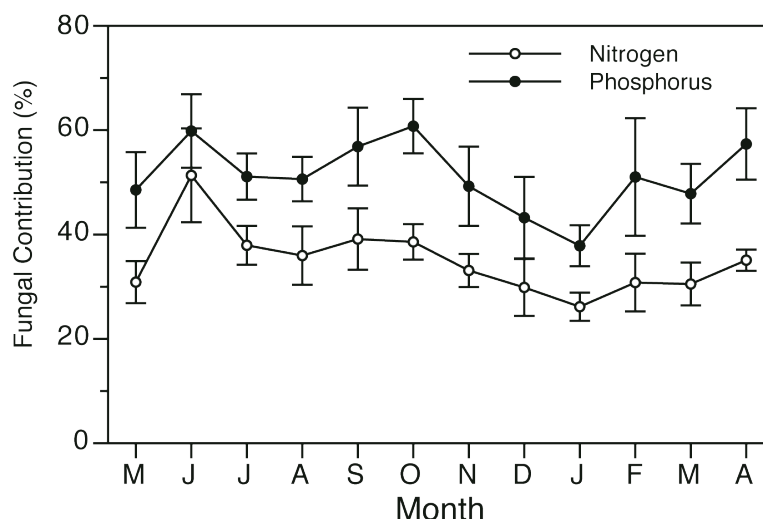


Figure 22. Estimated percentage of total detrital nitrogen and phosphorus immobilized by standing litter-associated fungal biomass. Symbols indicate the mean \pm 1SE.

Rates of Microbial Respiration. Rates of microbial respiration (CO_2 evolution) per gram of standing-dead plant litter and per square meter of marsh followed a similar pattern as corresponding rates of fungal production (Figure 23), with significant fluctuations in respiration rates being noted during the study period (ANOVA, $F_{11,59} = 7.76$ and 7.35 , respectively, $p < 0.001$). In accordance with both fungal biomass and production, a peak respiration rate was observed during the summer (June). Mean rates of respiration during the study period were positively related to both mean fungal biomass ($r = +0.73$) and rates of fungal production ($r = +0.90$, Pearson, $p < 0.05$), suggesting that most of the respiratory activity in standing-dead litter was due to inhabitant fungal decomposers. When integrated over the study period, estimated annual carbon flux from microbial respiration totaled 124 gC/m^2 (Table 4), representing $\sim 19\%$ of mean annual standing-dead litter mass ($643 \pm 104 \text{ gC/m}^2$). Assuming that all respiratory activity was

due to fungi, calculated annual fungal growth efficiencies (i.e., $GE = [\text{annual fungal production}] / [\text{annual fungal production} + \text{respiration}] \times 100$) was $42 \pm 3\%$ (Table 4).

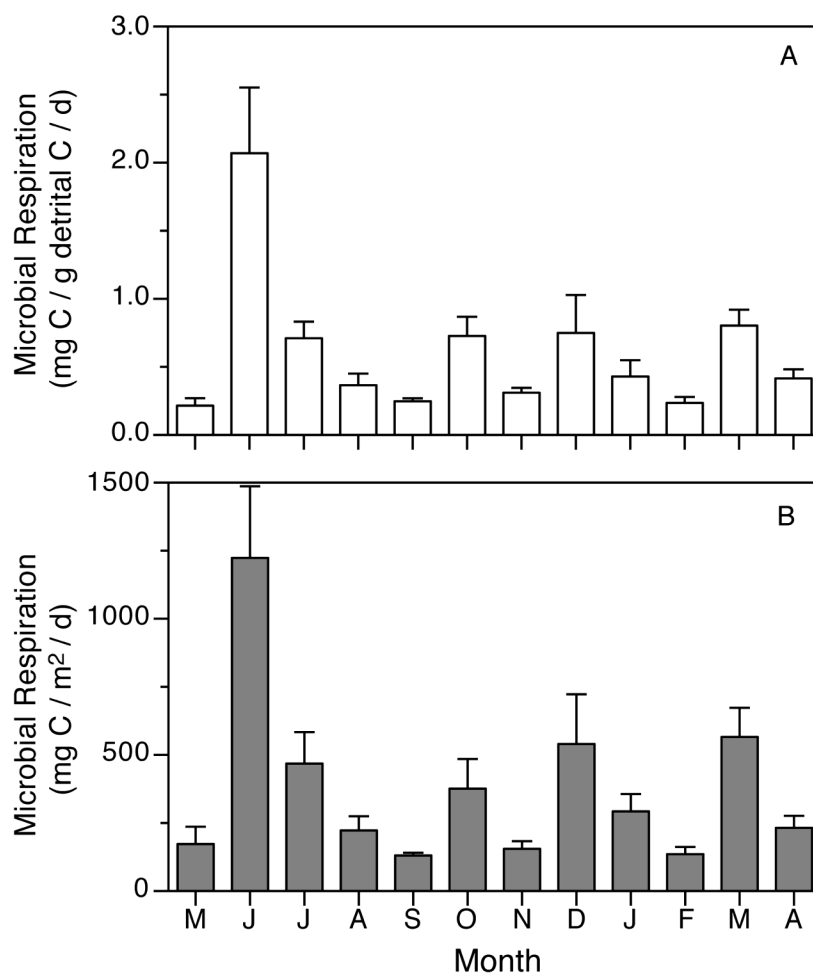


Figure 23. Rates of microbial respiration (A) per gram of standing detrital carbon and (B) per m² of marsh surface area in the *C. jamaicense* and *J. roemerianus* marsh stand during annual study period. Bars indicate the mean ± 1 SE.

Discussion

The results obtained in the present study reveal the presence of large quantities of standing-dead detritus at the Weeks Bay marsh site. Over the entire annual study period, monthly estimates of standing-dead plant litter mass on an areal basis averaged 643 gC/m², which significantly exceeded the corresponding estimate of aboveground living

plant biomass by ~1.65 times. Standing stocks of both living plant biomass and standing-dead litter mass did not significantly fluctuate during the annual study period, implying that a relatively steady pool of marsh plant carbon was produced that eventually accumulates and initially decomposes in the aerial standing-dead position. Standing-dead detritus also sequestered appreciable quantities of nitrogen and phosphorus, accounting for on average ~110% and 60% of the nitrogen and phosphorus standing stocks being observed in corresponding living plant biomass, respectively. These findings support prior published evidence establishing that standing-dead plant matter is a large and quantitatively important reservoir of detrital carbon and nutrients in freshwater marshes (Asaeda et al. 2002; Christensen et al. 2009), which ultimately forms the principle organic matter input that fuels the detritus-based food webs in these ecosystems (Moore et al. 2004; Hagen et al. 2012).

From an ecosystem perspective, the significant inputs of emergent plant detritus to freshwater marshes underscores that any comprehensive understanding of carbon flow and elemental cycling in these systems must include an examination of the natural microbial decay processes, since the growth and decay activities of marsh microbial communities are intimately coupled with, and are key drivers of, the transformation, assimilation, and mineralization of detrital organic matter. Despite the well-documented occurrence of appreciable standing-dead litter pools in freshwater marshes, most studies examining organic matter processing in these ecosystems have focused almost exclusively on structure and function of microbial communities occurring at or within the benthic sediments (e.g., Gutknecht et al. 2006; Rejmánková and Sirová 2007; Morrissey et al. 2014). This benthic-centered emphasis is likely grounded in the false perception

that the aerial standing-litter habitat is too harsh of an environment to support the significant growth and decay activities of decomposer microbes, and hence their overall contribution to ecosystem-level carbon and nutrient cycling is likely negligible. As a consequence, this restricted focus on benthic decay processes has guided much of our contemporary view of wetland biogeochemical cycles, where aerobic and anaerobic benthic microbial processes (i.e., bacteria) are often depicted as the sole pathways in ecosystem models describing marsh carbon and nutrient flow patterns (Mitsch and Gosselink 2007; Reddy and Delaune 2008; Kayranli et al. 2010).

In the present study, observations of notable fungal colonization, growth, and biomass accumulation in standing litter pools show that significant microbial transformation and mineralization of marsh plant detritus can occur during the initial standing-decay phase. At the Weeks Bay study site, appreciable rates of both fungal secondary production and microbial respiration per square meter of marsh were observed, with the highest values occurring during the warmer summer season, as expected. When integrated over the entire study period, rates of fungal secondary production in and microbial respiration (CO_2 evolution) from standing litter totaled 90 and 124 gC/m^2 , respectively, establishing that a sizeable fraction (~33%) of standing litter carbon pool (643 gC/m^2) flows into and through litter-inhabiting fungal communities on an annual basis. The significant correlation between rates of microbial respiration and litter-associated fungal biomass (ergosterol) and production rates implies that most of the observed respiratory flux from standing litter was due to fungal organisms (see also Kuehn et al. 2004). These findings highlight that microbial (fungal) decay processes in the standing litter are not minor but constitute an additional important pathway of carbon

flow that occurs before the collapse and benthic microbial decay of plant litter at and within the marsh sediments.

In the present study, findings of significant fungal contributions to ecosystem-level carbon flow may not be the exception, but instead may be the general rule within a variety of marine and freshwater systems. Although limited in number, prior studies applying fungal biomass and production methodologies in other freshwater and salt marsh systems are now providing convincing evidence of significant fungal contributions to marsh carbon cycling (Newell et al. 1995, 1996; Newell 2001a; Findlay et al. 2002; Gulis et al. 2006; Gessner et al. 2007; Kuehn 2008; Ohsowski 2008; Kuehn et al. 2011). Newell (2001a) was the first to roughly estimate ecosystem-scale fungal production in a subtropical salt marsh in Georgia. Using the acetate-to-ergosterol method (Gessner and Newell 2002), annual production of fungal decomposers associated with standing *Spartina alterniflora* litter totaled 230 gC/m², indicating that ~41% of the annual cordgrass production was converted into fungal biomass during standing litter decomposition. More recently, Kuehn (2008) reported annual fungal production rates associated with standing *T. angustifolia* litter in a north temperate freshwater marsh in Michigan. Annual rates of fungal production associated with standing-dead leaf-blades and stems totaled 70 and 45 gC/m²/y, respectively, which collectively was equivalent to roughly 10% of the yearly aboveground *Typha* production (1095 gC/m²/y). If fungal growth efficiencies were ~42%, as observed in the present study, then total fungal assimilation (production + respiration) of standing *T. angustifolia* litter could account for ~25% of the annual *Typha* production.

Similar areal production estimates have also been reported for fungal communities colonizing submerged plant detritus (Buesing and Gessner 2006; Ohsowski 2008), suggesting that fungal decomposers are likely to play an important role in the assimilation of plant litter carbon and nutrients during benthic litter decomposition (see also Kuehn et al. 2000; Komínková et al. 2000; Su et al. 2007). Buesing and Gessner (2006) estimated annual production rates of both bacterial and fungal communities associated with naturally-occurring benthic litter of *P. australis* in temperate lake-littoral marsh in Switzerland. Annual fungal production on submerged *P. australis* detritus totaled 93 gC/m²/y, indicating that ~15% of the annual aboveground *P. australis* production (603 gC/m²/y) was transformed into fungal biomass. However, in contrast to fungi, annual bacterial production was 7 times higher (661 gC/m²/y), providing supporting evidence that litter-associated bacterial decomposers, as expected, assume a more important role in carbon flow pathways once standing litter collapses to the marsh sediments.

In addition to carbon transformation in standing litter, fungal decomposers may also be a significant contributor to the transformation and assimilation of detrital nutrients, such as nitrogen and phosphorus. In the present study, estimates of living fungal biomass in standing litter remained fairly constant over the study period averaging 18 gC/m². Observations of appreciable biomass accrual in standing litter suggest that decomposer fungal communities are also capable of acquiring and immobilizing sources of detrital N and P, which would be required to meet the concomitant stoichiometric demands for growth and reproduction. This may be particularly true in standing litter decay systems where access to additional nutrients from exogenous sources is likely to be

more limited. Using a fungal stoichiometric ratio of $C_{106}N_{16}P_1$ (Beever and Burns 1980; Findlay et al. 2002; Gulis and Kuehn unpublished data), estimated nitrogen and phosphorus captured by fungi decomposers in the present study represented a maximum of 35 and 51% of the total detrital N and P in standing-dead litter, respectively. These results concur with other reports from both freshwater and salt marsh systems that fungal decomposers can sequester a sizable fraction of the detrital nitrogen and phosphorus pools in both standing and benthic litter compartments (Newell 1996; Newell and Porter 2000; Findlay et al. 2002; Van Ryckegem et al. 2006; Kuehn et al. 2011). For example, recently Kuehn et al. (2011) reported that fungal decomposers immobilized 60% and 83% of the total detrital N and P, respectively, during later stages of standing *T. angustifolia* leaf decay.

Quantifying detrital elemental pools and fluxes to litter decomposition products, such as CO_2 , microbial or consumer biomass, are important functional measures that clarify the role and contribution of microbial assemblages and consumers to ecosystem carbon and nutrient cycling (Gessner et al. 1999). The detritus-based nature of both freshwater and salt marsh ecosystems is widely known and accepted (e.g., Teal 1962; Mitsch and Gosselink 2007), where the accumulation and decomposition of plant matter is considered an important process for marsh sediment development and accretion, storage of carbon and nutrients, and microbial-based food resources for detrital consumers. Results obtained in the present study significantly extends our current conceptual understanding of marsh biogeochemical cycles by quantifying the magnitude of fungal secondary production and respiration at the ecosystem-level and the percentage of detrital carbon and nutrients that flow into and through decomposer fungal communities.

Hopefully, future researchers of freshwater marshes will recognize and appreciate, like researchers of other freshwater systems (Findlay 2010; Tank et al. 2010), that fungi can play key roles in the cycling of carbon and nitrogen and in mediating the flow of energy and nutrients to higher trophic levels.

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